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The Metabolism of [1-^{14}C]Glucose in an Enzyme System from *Propionibacterium*

By H. G. WOOD,* R. G. KULKA and N. L. EDSON
Department of Biochemistry, Medical School, University of Otago, New Zealand

(*Received* 4 *November* 1955)

Wood, Stjernholm & Leaver (1955) have proposed that propionic acid fermentation may involve the simultaneous occurrence of the Embden–Meyerhof cleavage and a second, undetermined, cleavage. The suggestion was based on the distribution of ^{14}C in the products obtained by fermenting [1-^{14}C]-glucose, [2-^{14}C]glucose, [6-^{14}C]glucose and [3:4-^{14}C]-glucose with cell suspensions. No matter which carbon atom of the glucose was labelled, there was considerable radioactivity in all the carbon atoms of the products. Although the widespread distribution of ^{14}C can probably be accounted for by the Embden–Meyerhof cleavage and subsequent randomization of ^{14}C via symmetrical C_4-dicarboxylic acids, and possibly via a symmetrical C_3 intermediate, the occurrence of a pathway other than the Embden–Meyerhof cleavage in whole cells is indicated by the following considerations: (1) The carbon dioxide formed from [1-^{14}C]glucose had a higher activity than any of the carboxyl groups of the end products and a much greater activity than the carbon dioxide from [6-^{14}C]glucose; (2) the acetate from [1-^{14}C]glucose had a higher activity than that from [6-^{14}C]glucose; (3) the peculiar distribution of ^{14}C in the products from the other types of labelled glucose suggested the existence of an unknown metabolic pathway.

In order to obtain more detailed information about the mechanism it appeared desirable to have a cell-free enzyme preparation which could carry out the whole or part of the propionic acid fermentation. It has been found that such an enzyme system can be obtained by sonic disintegration of propionic acid bacteria. The preparations form propionate, succinate, acetate and carbon dioxide from [1-^{14}C]glucose and the ^{14}C is randomized into all positions of the products. The enzyme system of *Propionibacterium shermanii* is remarkable in that it promotes a net uptake of carbon dioxide when glucose is metabolized.

EXPERIMENTAL

The organisms were *Propionibacterium arabinosum* (34W) and *Pr. shermanii* (52W) used previously by Wood *et al.* (1955) and maintained in lactate–glucose stab cultures. These strains were selected for investigation because they give quite different proportions of propionic acid, acetic acid and CO_2 in the fermentation of glucose (Wood & Werkman, 1936). The bacteria were grown for 3–5 days at 30° in 0·05M potassium phosphate buffered medium (pH 6·8), 1 l. of which contained 5·0 g. of glycerol, 5·0 g. of Difco yeast extract and added vitamins (1·0 mg. of calcium pantothenate, 1·0 mg. of thiamine hydrochloride, 0·01 mg. of biotin). For use in experiments they were transferred through 10 and 75 ml. of the glycerol medium and then cultivated in 2 l. conical flasks containing 1·5 l. of medium. The flasks were plugged with cotton wool. The cells were harvested by centrifuging and washed twice with 10 vol. of distilled water. The yields (wet wt.) were about 4 g. of *Pr. arabinosum* and 1 g. of *Pr. shermanii*/l. of medium.

Preparation of cell-free extracts. The wet cells were suspended in 1·5–5 times their weight of ice-cold 0·04–0·1M potassium phosphate buffer, pH 7·0. Batches (20–25 ml.) of the suspensions were treated for 1·5–2 hr. in a Raytheon 9 kc./sec. sonic disintegrator. The temperature of the suspension was maintained at 0–1° by placing the cup assembly in a cold room at 2–3° and circulating ice water through the cooling chamber at the rate of 1·8 l./min. The extracts were clarified by centrifuging for 0·5 hr. at 20 000 *g* in a refrigerated centrifuge operating at −4°. The brown supernatant solutions were quite clear but showed a pronounced Tyndall effect. When the original suspension contained sufficient phosphate buffer the pH of the extracts was 6·5–6·9. Extracts which were frozen and stored at −40° retained their activity for 8 weeks. There was about 50% loss of activity when they were freeze-dried.

Materials. [1-^{14}C]Glucose was obtained from the National Bureau of Standards, Washington, D.C.; $NaH^{14}CO_3$ was obtained from the Radiochemical Centre, Amersham, England. Adenosine triphosphate (ATP) was either the cryst. disodium salt (Sigma Chemical Co., St Louis, U.S.A.) or a solution of the sodium salt prepared from dibarium ATP (Pabst Laboratories, Milwaukee, U.S.A.) by ion exchange on Amberlite IRC 50 (sodium form). Hexose diphosphate (HDP) was used as a solution of the sodium salt prepared from the dibarium salt (Schwarz Laboratories, Inc., New York, U.S.A.) by removal of Ba on a Dowex 50 column (acid form) and by neutralization with NaOH. Diphosphopyridine nucleotide (DPN) was the chromatographically purified product of Schwarz Laboratories, Inc., New York. Sodium pyruvate was prepared by the neutralization with sodium bicarbonate of pyruvic acid which had been freshly distilled *in vacuo*.

Analytical methods

Carbon dioxide evolution during fermentation, and bound CO_2, were measured manometrically either in conventional Warburg vessels or in 200 ml. vessels. Net

* Fulbright Visiting Professor. Permanent address: Department of Biochemistry, Western Reserve University, Cleveland, Ohio, U.S.A.

utilization of CO_2 was measured by determining the bound CO_2 at zero time, the CO_2 liberated during fermentation, and the bound CO_2 at the end of the experiment. There was a large evolution of gas from $NaHCO_3$ when H_2SO_4 was tipped into the vessels, which were not shaken until most of the bound CO_2 had been liberated. When ^{14}C in the CO_2 was to be measured, the vessel was removed after the manometric determination of bound CO_2, and alkali was added quickly to the centre well and the vessel was closed again in order to collect a sample of the CO_2.

Glucose was determined colorimetrically by the methods of Somogyi (1945) and Nelson (1944).

Isolation of the products of fermentation. The acidified fermentation mixture was centrifuged and the protein precipitate washed with water. The combined supernatant and washings were extracted continuously with diethyl ether for 2 days. The water–ether mixture in the extraction flask was titrated with NaOH and then evaporated to dryness. The acids were separated on a Celite column (Swim & Krampitz, 1954). The succinic acid fraction was oxidized with acid permanganate to remove any lactic acid that might have been present, and re-extracted with ether and then sublimed at 115° under vacuum. The volatile-acid fractions were distilled in steam and titrated. Carrier acids were added when this was found to be necessary, on the basis of the titrations.

Degradations. Acetic and propionic acids were degraded by the Schmidt reaction (Phares, 1951). Formate was oxidized with mercuric oxide (Osburn, Wood & Werkman, 1933).

Succinate was converted into propionate and CO_2 with a suspension of *Micrococcus lactilyticus*. The bacteria were grown anaerobically at 37° under N_2+CO_2 (95:5, v/v) in a medium containing 2·0% Difco peptone, 1·0% Difco-yeast extract, 2·0% sodium lactate, 0·5% NaCl, 0·25% K_2HPO_4 and 0·1 mg. % thiamine hydrochloride. The cells were harvested when they liberated gas vigorously (24 hr.) and were washed twice with 0·1 M potassium phosphate buffer, pH 5·9. The succinate was fermented in a 200 ml. Warburg flask under nitrogen at 37°. The reaction mixture contained ~0·03 M succinate, 0·1 M potassium phosphate buffer (pH 5·9), 0·005 M-$MgSO_4$, 0·01 M-NaF and 0·7% (wet wt.) of cells. The gas evolution was observed manometrically until the rate decreased sharply, when the bound CO_2 was liberated by addition of H_2SO_4 from the side-bulb. A control fermentation with the cells in the absence of succinate gave a small amount of acetate but no propionate. The volatile acids were distilled in steam, and the propionic acid was separated on a Celite column and subsequently degraded by the Schmidt reaction.

The accuracy of each degradation was checked by comparing the sum of the activities in the degradation fractions with the total activity of the compound determined on the CO_2 after combustion with chromic acid (Van Slyke & Folch, 1940).

Measurement of radioactivity. All samples were plated on filter paper as barium carbonate and counted with an end-window Geiger–Müller counter. The counts were corrected for background and for self-absorption (to infinite thinness).

RESULTS

Manometric experiments

The enzyme systems from the two species of *Propionibacterium* had very different properties (Table 1). The extract of *Pr. arabinosum* required a combination of substrates (glucose, HDP and pyruvate), and also ATP and DPN, for maximal activity. Even with these additions (Table 1, no. 7)

Table 1. *Fermentation of glucose, hexose diphosphate and pyruvate by cell-free extracts of* Propionibacterium

Warburg vessels contained 1·60 ml. of medium, pH 6·9; gas, CO_2; temp. 30°. Final concentrations of constituents in the fermentation mixtures were: 0·05 M glucose, 0·01 M HDP, 0·02 M pyruvate, 0·001 M ATP, 0·0005 M DPN, 0·12 M-$NaHCO_3$. The bacterial extract was added from the side bulb and readings began after 10 min. The extract in each vessel represented 0·16 g. of *Pr. arabinosum* and 0·13 g. of *Pr. shermanii* (both wet wt.). Vessels contained ATP and DPN unless omission is indicated.

		μl. of CO_2 evolved/hr.*					
		Pr. arabinosum (34 W)			*Pr. shermanii* (52 W)		
Time interval (hr.)		0–1	1·5–2·5	2·5–3·5	0–1	4–5	27–28
Vessel no.	Substrate added						
1	Glucose	−29	−10	−10	60	50	44
2	HDP	58	37	7	19	11	16
3	Pyruvate	−2	2	0	44	−5	4
4	Glucose, HDP	54	55	5	54	56	42
5	Glucose, pyruvate	8	7	−2	131	57	48
6	HDP, pyruvate	222	32	5	86	13	16
7	Glucose, HDP, pyruvate	668	490	172	162	74	47
8	Glucose, HDP, pyruvate (ATP and DPN omitted)	328	97	0	123	53	39
9	Glucose, HDP, pyruvate (DPN omitted)	605	207	19	162	63	35
10	Glucose (ATP and DPN omitted)	—	—	—	35	31	36
11	None	45	16	11	56	38	15

* The values are the averages of two tests, each with different extracts, and have been corrected for the value obtained in the absence of added substrate.

the activity decreased rapidly, and in 4–5 hr. there was no further formation of CO_2 or acid. In contrast the extract of *Pr. shermanii* fermented glucose at a constant rate in the absence of added ATP and DPN (Table 1, no. 10). The rate was somewhat higher when ATP and DPN were added (Table 1, no. 1), and the activity was greater with the combined substrates (Table 1, no. 7), but after 5 hr. the rate fell almost to that of glucose alone. The enzyme system of *Pr. shermanii* was remarkably stable, fermenting glucose at a constant rate for 30 hr. This extract metabolized pyruvate very actively as a single substrate, all the pyruvate disappearing in 6 hr. (negative nitroprusside test).

With *Pr. shermanii* the sum of the activities in the first hour on the individual substrates (glucose, pyruvate or HDP) was almost equal to the activity on the combination of the three. In the presence of single substrates the extract of *Pr. arabinosum* acted only on HDP, and no combination of two substrates gave as much fermentation as the three combined. When the concentrations of ATP and HDP were double those in Table 1 (no. 7), a higher rate of activity was maintained for a longer time. If more ATP was added to no. 7 when the fermentation rate had fallen to a low value, the activity increased again. Thus it seems probable that one function of the HDP is to maintain a supply of ATP, the concentration of which limits the fermentation. Pyruvate may serve as a hydrogen acceptor: it is still present when the reaction stops (positive nitroprusside test) and doubling the amount of glucose does not increase the yield of CO_2.

The extract of *Pr. shermanii* is remarkable in bringing about a net uptake of CO_2 when glucose is fermented. A net uptake of CO_2 in the propionic acid fermentation has been obtained before only with polyols such as glycerol, erythritol, ribitol or mannitol (Wood & Leaver, 1953; Wood & Werkman, 1940). Under conditions corresponding to those of Table 1 (no. 1), 91·1 μmoles of CO_2 were evolved during the fermentation and 78·6 μmoles of bound CO_2 were released on addition of acid at 30 hr. At zero time the bound CO_2, determined by addition of acid, was 201·3 μmoles. Accordingly, the net uptake was 31·6 μmoles of CO_2. Since 80 μmoles of glucose were added, 40 μmoles of CO_2 were utilized/100 μmoles of glucose. This substantial uptake of CO_2 is comparable with that previously obtained with glycerol (Wood & Werkman, 1938). The extract of *Pr. arabinosum* did not cause net utilization of CO_2 under the same conditions.

Table 2. *Products of fermentation of* [1-^{14}C]*glucose by extracts of* Propionibacterium

Fermentations were conducted in 200 ml. Warburg vessels containing 32 ml. of reaction mixture with the bacterial extract initially in one side bulb and H_2SO_4 in the other: gas, CO_2; temp. 30°. Time, 33·5 hr. with *Pr. shermanii* and 6·5 hr. with *Pr. arabinosum*. The final concentrations of the constituents were: with *Pr. arabinosum*, 0·05 M [1-^{14}C]glucose (40300 counts/min./m-mole); 0·01 M HDP; 0·02 M pyruvate; 0·0019 M ATP; 0·00025 M DPN; 0·12 M-$NaHCO_3$; bacterial extract corresponding to 2·6 g. (wet wt.) of cells; with *Pr. shermanii* an extract corresponding to 2·25 g. (wet wt.) of cells was mixed with the same constituents except HDP, pyruvate and DPN, which were omitted. To determine the initial bound CO_2 control vessels were set up with one-quarter the amount of the constituents, and the bacterial extract and H_2SO_4 were tipped simultaneously. The organic acids in the ether extracts from these control vessels were determined. The values ($\times 4$) for the organic acids in the control vessels were 0·069 m-mole of propionate and 0·043 m-mole of succinate for *Pr. shermanii* extract, and 0·044 m-mole of propionate and 0·011 m-mole of succinate for *Pr. arabinosum* extract. Formate and acetate values were not obtained. No correction has been made for the acids initially present in the extracts. *Pr. shermanii*: recovery of carbon in the products from the fermented glucose (1·27 m-moles) is 72%; oxidation/reduction = 1·28; added radioactivity accounted for, 90%; CO_2 liberated during fermentation (equivalent of acid formation less CO_2 fixed), 2·64 m-moles. *Pr. arabinosum*: added radioactivity accounted for, 85%; CO_2 liberated during fermentation (equivalent of acid formation plus CO_2 produced), 1·58 m-moles.

	Pr. shermanii					*Pr. arabinosum*			
Product	m-moles	Counts/min./m-mole	Total counts/min.	Percentage added counts/min.	m-moles/100 m-moles of glucose fermented	m-moles	Counts/min./m-mole	Total counts/min.	Percentage added counts/min.
Propionate	0·412	14 700	6 050	9·4	32·4	0·591	6 060	3 580	5·6
Acetate	0·434	10 800	4 670	7·3	34·2	0·434	2 460	1 070	1·7
Formate	0·072	2 180	157	0·2	5·7	0·160	195	31	0·05
Succinate	0·85	16 560	14 100	21·9	66·9	0·125	11 000	1 380	2·2
CO_2	−0·155	526*	6 310*	9·8	−12·2	0·40	183*	2 200*	3·4
Carbon in residue from ether extraction	9·54	2 780	26 500	41·2	—	11·4	4 080	46 500	72·2

* Approximately 12 m-moles of gaseous CO_2. $NaHCO_3$ and dissolved CO_2 were estimated to be present in the vessels after fermentation.

The addition of sodium fluoride (0·03 M final concentration) inhibited the fermentations conducted with *Pr. shermanii* and *Pr. arabinosum* under the conditions of Table 1, in nos. 1 and 7 respectively. Carbon dioxide evolution was almost completely inhibited from the beginning in the *Pr. shermanii* fermentation; and the inhibition was about 50 % with *Pr. arabinosum* in the first 35 min. (observations not continued).

Products from [1-^{14}C]*glucose and the distribution of* ^{14}C

Fermentations of [1-^{14}C]glucose were carried out with extracts of *Pr. arabinosum* and *Pr. shermanii*, HDP and pyruvate being present in addition to glucose in the former extract. The conditions of the experiments, yields of products and the recovery of ^{14}C are shown in Table 2. The yield of products per 100 m-moles of fermented glucose has been calculated for the *Pr. shermanii* fermentation in order to present the data in a form comparable with that of previous experiments. Recovery of the carbon of the fermented glucose was only 72 % and the oxidation/reduction balance was 1·28. Probably some product other than those determined was formed by the extract. The unfermented glucose (0·33 m-mole) was equivalent to 13 300 counts/min., but 26 500 counts/min. were found in the residue from ether extraction. The identity of this unknown product is being investigated. The most noteworthy point regarding the yield of products is the very large quantity of succinate (66·9 m-moles/100 m-moles of glucose), which is much greater than the amount (15 m-moles/100 m-moles of glucose) found by Wood & Werkman (1936) with proliferating cells of *Pr. shermanii*. The high yield of succinate in the enzymic fermentation accompanies the net utilization of CO_2 (12·2 m-moles/100 m-moles of glucose). A similar relationship was observed previously in glycerol fermentations (Wood & Werkman, 1938). It is to be noted also that 9·8 % of the added ^{14}C was converted into CO_2, showing that the enzyme system, like the intact cells, converts C-1 of glucose into CO_2.

The extract of *Pr. arabinosum* converted much less of the ^{14}C of glucose into propionate, acetate, succinate and CO_2 than the extract of *Pr. shermanii* (13·0 % compared with 48·6 %). Apparently a considerable portion of the products arose from HDP and pyruvate, and much of the glucose carbon remained in the residue after ether extraction (72 % of the ^{14}C), possibly as phosphate esters.

The distribution of ^{14}C in the products is shown in Table 3, in which data obtained by Wood *et al.* (1955) with resting cells have been included for comparison. It will be seen that the enzyme preparations randomize ^{14}C from C-1 of glucose into all positions of the products. Any comparison

Table 3. *Distribution of* ^{14}C *in the products of fermentation of* [1-^{14}C]*glucose*

The conditions of the experiments are the same as in Table 2.

Specific activity as percentage of the specific activity in C-1 of glucose

Organism	CO_2	Propionate					Succinate			Acetate			
		Formate	CH_3 –	CH_3 – CO_2H	Sum	Combustion ($C \times 3$)	$(CH_2 - CO_2H)_2$	Sum	Combustion ($C \times 4$)	CH_3 – CO_2H	Sum	Combustion ($C \times 2$)
Pr. shermanii	1·3	5·4	17·2	18·3 2·0	37·5	36·5	16·4	37·6	41·1	14·1	12·3 26·4	26·8
Pr. arabinosum	0·5	0·5	7·7	7·3 1·8	16·8	15·0	10·2	27·0	27·3	4·7	2·7 7·4	6·1
**Pr. shermanii*	23·6	—	13·2	13·2 10·9	—	—	12·7	9·1	—	—	— —	—
	12·2	—	10·5	9·3 4·9	—	—	7·3	3·7	—	9·5	2·1 —	—
**Pr. arabinosum*	19·7	—	14·2	15·7 8·1	—	—	15·5	4·5	—	15·1	5·2 —	—

* Data from Table 1 of Wood *et al.* (1955).

between experiments with extracts and intact cells must take cognizance of the differences in experimental conditions. In the experiment with extracts the gaseous phase was CO_2, whereas with cells it was nitrogen and the CO_2 formed from glucose was absorbed in alkali. Thus the ^{14}C in the CO_2 arising from glucose was greatly diluted by $^{12}CO_2$ in the extracts, and the specific activity was low. This in turn caused a decrease in the specific activity in the carboxyl groups of the propionate and succinate since CO_2 fixation introduced carbon with low radioactivity. If this difference in the experiments is taken into account, the results with the extracts and the intact cells are quite similar. The radioactivity in the products formed by the *Pr. arabinosum* extract was diluted further by metabolites arising from HDP and pyruvate.

Formate showed considerable radioactivity in the experiment with *Pr. shermanii* extract. Formate is not usually a product in the propionic acid fermentation, but Wood & Leaver (1953) have noticed its formation, especially from polyols.

Fermentation of pyruvate and fixation of CO_2

Fermentation of pyruvate in the presence of radioactive bicarbonate was conducted with an extract of *Pr. shermanii* to determine the products and the extent of CO_2 fixation (Table 4). An odour of diacetyl was noticed and for this reason diacetyl and acetoin were determined together in a portion of the extract by oxidation with ferric chloride, distillation and estimation of the resulting diacetyl (Westerfield, 1945). Usually acetoin and diacetyl are not formed by the propionic acid bacteria, but a considerable amount was produced with the extract. The other products were propionate, acetate, succinate and CO_2. The CO_2 production was quite substantial in contrast to the net uptake of CO_2 which occurs in the presence of glucose (Table 2). Nevertheless, there was a large fixation of CO_2 since the propionate contained 14 300 counts/min./m-mole as compared to the CO_2 which had 24 200 counts/min./m-mole at the termination of the experiment. Although the propionate was not degraded it is reasonably certain that all the activity was in the carboxyl group (Wood & Leaver, 1953). This means that about half the carboxyl groups of the propionate must have originated from CO_2.

Table 4. *Fermentation products formed from pyruvate by an extract of* Pr. shermanii (52W) *in the presence of* $NaH^{14}CO_3$

The fermentation was carried out in a 200 ml. Warburg vessel containing 8 ml. of reaction mixture with the bacterial extract in one side bulb and H_2SO_4 in the other: gas, CO_2; temp. 30°, time, 20 hr. Final concentrations: 0·08 M pyruvate, 0·0019 M ATP, 0·12 M-$NaH^{14}CO_3$. The extract represented 0·56 g. (wet wt.) of cells. Pyruvate added was 0·64 m-mole. Carbon recovery in the products was 93·7%; oxidation/reduction = 1·10; the CO_2 in the gaseous phase after fermentation contained 24 200 counts/min./m-mole. Propionate contained 14 300 counts/min./m-mole.

	Amount formed	
Product	m-mole	m-moles/100 m-moles of pyruvate
Propionate	0·138	21·6
Acetate	0·212	33·0
Formate	0·029	4·5
Succinate	0·052	8·1
CO_2	0·463	72·3
Acetoin + diacetyl	0·066	10·3

DISCUSSION

The results demonstrate that it is possible to extract from *Pr. shermanii* (52W) a soluble-enzyme system, which contains all the enzymes necessary for conversion of glucose or pyruvate into propionate, succinate, acetate and carbon dioxide, and for the production of carbon dioxide from C-1 of glucose. The remarkably widespread distribution of ^{14}C into all the carbon atoms of the products formed by this enzyme system during the fermentation of [1-^{14}C]glucose is comparable with that observed in the intact cells. The preparation contains a very active system for the fixation of carbon dioxide and for the production of succinate. For this reason it appears to be very suitable for a study of the complete propionic acid fermentation.

Although the extract of *Pr. arabinosum* (34W) has many similar properties, it is inactive with glucose as the sole substrate and demands, in addition, HDP and pyruvate. It appears to contain enzymes destroying the ATP that is needed in the fermentation; and it may also lack some enzymes required for glucose breakdown.

The formation of diacetyl is interesting because the propionic acid fermentation is not known to involve acetaldehyde. Since acetaldehyde is thought to be an intermediate of acetoin formation, it is possible that acetaldehyde or a derivative may participate in the propionic acid fermentation.

SUMMARY

1. Soluble-enzyme systems, free from particulate matter, have been extracted from *Propionibacterium shermanii* and *Propionibacterium arabinosum* by sonic disintegration of the cells.

2. The extracts ferment glucose or pyruvate. The fermentation of glucose by *Pr. shermanii* extract is unique in that there is a net uptake of carbon dioxide.

3. The products of fermentation of [1-^{14}C]-glucose have been isolated and degraded. The enzyme system randomizes ^{14}C into all positions of the products. Carbon dioxide is produced from C-1 of glucose, and this may occur by some pathway additional to the Embden–Meyerhof cleavage.

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The Influence of Acetamide on Citrate Accumulation after Fluoroacetate Poisoning

By S. GITTER*

Department of Biochemistry, University of Oxford

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Up to 1949 only symptomatic therapy against fluoroacetate poisoning had been attempted, but no satisfactory results could be obtained by several workers. Sedatives and anticonvulsants gave no protection (Foss, 1948).

Bartlett & Barron (1947) and Saunders & Stacey (1948) considered the action of fluoroacetate to be a specific inhibition of acetate metabolism; this led Chenoweth, Scott & Seki (1949) to ascertain whether acetate would protect the animal against fluoroacetate, but little or no protection of the intact rabbit could be obtained by them even after very high doses of sodium acetate. Only by combining acetate with ethanol could Tourtelotte & Coon (1949) obtain protection in mice, and good results were also reported (Hutchens, Wagner, Podolsky & McMahon, 1949) by the use of ethanol alone. Chenoweth, Kandel, Johnson & Bennett (1951) then reported monoacetin as the most efficient protective agent against fluoroacetate poisoning.

Gitter, Blank & Bergmann (1953*a*) showed that acetamide protected white rats against fluoroacetate poisoning when given simultaneously or up to 24 hr. before the dose of fluoroacetate. Acetamide did not protect when given 30 min. or more after the administration of fluoroacetate, even before the convulsions had started.

The protective results obtained suggested that acetamide might belong to those two-carbon substances, which, according to Peters (1952*a*), penetrate into the mitochondria and are consequently able to interfere with the formation of the fluorotricarboxylic acid. The influence of acetamide on the accumulation of citrate after the administration of fluoroacetate has been studied. The effects of monoacetin (Chenoweth *et al.* 1951), propionamide and butyramide on citrate accumulation were also tested. A related problem was the low toxicity which we had observed for fluoroacetamide (Gitter, Blank & Bergmann, 1953*b*), and the effect of this substance on citrate accumulation has also been examined.

* Present address: The Rogoff Medical Research Institute, Beilinson Hospital, Petah Tiqva, Israel.

EXPERIMENTAL

Materials

Acetamide. Acetamide (B.D.H.) was recrystallized and an aqueous solution neutralized by addition of 0·1 N-NaOH. The neutralized solution, containing 250 mg. of acetamide/ml., was injected subcutaneously to rat in doses of 125 mg./100 g. body wt.

Sodium fluoroacetate. This was prepared in aqueous solutions of 5 and 10 mg./ml.; 0·1 ml./100 g. body wt. was injected intraperitoneally.

Monoacetin. This was given undiluted by subcutaneous or intraperitoneal injection in a dose of 0·5 ml./100 g.

Propionamide and butyramide (a mixture of equal quantities of n- *and* iso-*butyramide).* These were injected in a similar manner and dosage as for acetamide (125 mg./100 g. body wt.).

White male rats, weighing 120–240 g., and female guinea pigs were used. The animals were killed by decapitation,

and extracts of brain, heart and kidney were prepared within 10 min. by grinding the tissues with 8% trichloroacetic acid in an ice-cold mortar. All experiments with acetamide were performed on animals starved for 16–24 hr. before fluoroacetate treatment.

Citrate was determined in the extracts by the method of Pucher, Sherman & Vickery (1936) as modified by Buffa & Peters (1950).

RESULTS

Citrate in rat brain, heart and kidney after treatment with acetamide or monoacetin

White rats treated only with acetamide showed no toxic signs, though in some cases macroscopic haematuria occurred.

The citrate levels in the organs of these animals were much higher than normal, although they did not reach the level of fluoroacetate-treated animals. Table 1 shows the results obtained 60 and 120 min. after acetamide and monoacetin had been given. Normal values are also shown. These are in agreement with the original findings of Buffa & Peters (1949), Potter, Busch & Bothwell (1951) and Lindenbaum, White & Schubert (1951).

With acetamide, maximum citrate values were obtained after 2 hr., and the level then remained approximately constant for several hours. Monoacetin was also found to increase the citrate contents of the organs within 2 hr. to an even greater extent than acetamide, particularly in the heart.

Effect of injecting fluoroacetate and acetamide on citrate levels of tissues

Accumulation of citrate after administration of fluoroacetate, with and without protection from acetamide, has been studied. Extracts of brain, heart and kidney of male rats, 35 and 60 min. after injection of sodium fluoroacetate in doses of 5 and 10 mg./kg., were analysed for citrate. Acetamide was always given 2 hr. before sodium fluoroacetate in a single subcutaneous injection. Each time a citrate ratio was calculated, defined as the percentage of the citrate content of an organ of an acetamide-protected animal with respect to the content in the same organ of a non-protected animal, both after fluoroacetate treatment.

The results are shown in Table 2. In all cases, acetamide was found to have no appreciable effect on citrate accumulation in the brain after fluoroacetate. In the heart, however, which accumulates higher concentrations of citrate than brain, acetamide clearly reduces the citrate ratio. A similar but more pronounced effect is seen in the kidney, where acetamide strongly counteracts the citrate accumulation.

Table 1. *Citrate concentrations in rat tissues after injection of acetamide, monoacetin or fluoroacetate*

Citrate concentrations are given in μg./g. of fresh tissue. Na fluoroacetate injected at 1 mg./100 g. body wt. All citrate concentrations are individual values, except in last column where 3–4 rats were used.

	Normal rats	Acetamide-treated after		Monoacetin-treated after		Fluoroacetate-treated after
		60 min.	120 min.	60 min.	120 min.	60 min.
Brain	30	52	118	187	208	279±63
Heart	44	88	135	207	400	1277±291
Kidney	27	131	121	125	206	1291±245

Table 2. *Citrate contents of tissues after injection of fluoroacetate and effects of acetamide injections (125 mg./kg. body weight)*

Citrate contents are expressed as μg./g. of fresh tissues, and are average values ± standard deviation.

Time after injection of Na fluoroacetate (min.)	Amount of Na fluoroacetate injected (mg./kg.)	No. of expts.		Brain	Heart	Kidney
35	5	4	With acetamide	233±85	342±132	254±55
			Without acetamide	240±60	576±108	647±220
			Citrate ratio	97	59	42
60	5	3	With acetamide	222±60	353±36	228±35
			Without acetamide	283±26	1147±76	816±180
			Citrate ratio	81	31	29
35	10	3	With acetamide	278±50	454±24	369±126
			Without acetamide	285±65	1209±145	1033±130
			Citrate ratio	97	38	37
60	10	3	With acetamide	274±16	710±119	627±225
			Without acetamide	279±63	1277±291	1291±245
			Citrate ratio	98	56	52

Table 3. *Effect of acetamide injections on the citrate levels of guinea-pig tissues after fluoroacetate poisoning*

	Citrate contents of tissues (μg./g. fresh weight)					
	75 min. after sodium fluoroacetate (2 mg./kg.)			150 min. after sodium fluoroacetate (4 mg./kg.)		
	With acetamide	Without acetamide	Citrate ratio	With acetamide	Without acetamide	Citrate ratio
Brain	91	141	65	223	295	76
	127	225	56	287	325	89
Heart	540	821	66	895	675	132
	616	743	83	—	—	—
Kidney	223	784	35	432	1113	39
				392	1251	31

Doubling the dose of fluoroacetate or increasing the period of fluoroacetate poisoning from 35 to 60 min. caused the citrate contents of the unprotected animals to rise in heart and kidney but had no effect on the level in brain. Acetamide prevented any further increase of the citrate accumulation in heart and kidney between 35 and 60 min. after a fluoroacetate injection of 5 mg./kg. body weight.

Influence of acetamide on the accumulation of citrate caused by sodium fluoroacetate in guinea pigs

It is of importance to know whether or not the effect of acetamide in decreasing citrate accumulation after fluoroacetate is a specific reaction for white rats. Doses of 2 and 4 mg./kg. respectively were therefore injected into eight female guinea pigs, half of which had received acetamide protection. The dose of 2 mg./kg. is well above the LD_{100} for these animals. The latent period for guinea pigs is longer than for rats and 75 and 150 min. were therefore allowed to elapse between the administration of fluoroacetate and the preparation of the extract. Results are given in Table 3. The animals killed after 75 min. had not convulsed, but those killed after 150 min. had all suffered one or more convulsions.

It is seen that the reduced citrate ratio after acetamide injection is found in guinea pigs as well as in rats, especially in kidney. It is not definite in the heart, however, where the values obtained after administration of sodium fluoroacetate (4 mg./kg.) were inconsistent. Brain, however, showed a tendency towards a lower citrate ratio, although again the absolute values for citrate accumulation found in the brain of this species are below those of heart and kidney.

Comparison of the effects of acetamide and monoacetin

Because of the similarity of the effects produced by acetamide and monoacetin (see Table 1), the effects of both compounds on the citrate accumulation, provoked by sodium fluoroacetate administered in a dose of 10 mg./kg., have been compared. The citrate ratios obtained by administering these substances is given in Table 4. A similarity is found between the results obtained with each compound; however, monoacetin was also found to reduce the citrate ratio in the brain of white rats.

Table 4. *Citrate ratios with acetamide and monoacetin after administration of sodium fluoroacetate (10 mg./kg.)*

	Citrate ratio after administration of			
	Acetamide		Monoacetin	
	35 min. (%)	60 min. (%)	35 min. (%)	60 min. (%)
Brain	97	100	66	57
Heart	38	56	73	61
Kidney	37	52	35	27

Effect of fluoroacetamide

This fluoro derivative shows a comparatively low toxicity. A dose of 30 mg./kg. is needed to produce the usual effects of fluoroacetate poisoning with convulsions and ultimate death of the animal. A dose of 10 mg./kg. provoked no visible toxic effects, but 20 mg./kg. appeared to be slightly above the LD_{50}.

From Table 5 it is seen that 20 mg. of fluoroacetamide/kg. produced about the same citrate accumulation as 5 mg. of sodium fluoroacetate/kg. when the animals were protected by acetamide (cf. Table 2). Administration of acetamide to fluoroacetamide-treated animals did not result in any appreciable change of the citrate ratio.

As fluoroacetamide appeared to combine both the toxic action of fluoroacetate and the protective action of acetamide, although the doses of fluoroacetamide were far below those used for acetamide, lower doses of acetamide were tested to determine whether they would also influence the citrate ratio. Rats were injected with 12·5 mg./100 g. body weight instead of the usual 125 mg./100 g. The

Table 5. *Citrate contents of rat tissues* 60 *min. after injecting with fluoroacetamide*

Fluoroacetamide injected: (*a*) 10 mg./kg.; (*b*) 20 mg./kg.

		Citrate (μg./g. fresh wt.)		
		With acetamide	Without acetamide	Citrate ratio
Brain	*a*	205	202	101
	b	232	285	81
Heart	*a*	335	353	95
	b	400	391	102
Kidney	*a*	152	194	78
	b	210	201	105

Table 6. *Citrate contents of rat tissues* 35 *min. after injection of sodium fluoroacetate* (10 *mg./kg.*)

Acetamide injected: (*a*) 125 mg./100 g.; (*b*) 12·5 mg./100 g.

		Citrate (μg./g. fresh wt.)		
		With acetamide	Without acetamide	Citrate ratio
Brain	*a*	290	306	94
	b	310	375	83
Heart	*a*	457	1161	40
	b	950	1200	79
Kidney	*a*	411	1032	40
	b	507	965	52

results are shown in Table 6. A reduction of citrate in kidney was obtained by this small dose of acetamide, but the effect was less obvious in heart. As before, brain-citrate levels did not respond to either dose of acetamide. These results with low doses of acetamide show that more detailed pharmacological investigations are needed in order to find the minimum effective dose.

Activity of other amides

Propionamide, in doses of 125 mg./100 g. body weight, did not have any appreciable influence on citrate accumulation. The citrate ratio for heart was 100, for brain 80, and for kidney 90, 60 min. after administration of sodium fluoroacetate in a dose of 10 mg./kg.

Butyramide, however, in doses of 125 mg./100 g. body weight, which alone did not influence the citrate level in brain, lowered the citrate accumulation in heart and kidney, the citrate ratios being 71 and 72% respectively. It is not known whether differences exist between *n*- and *iso*-butyramide, or whether butyramide as such has the protective action, or whether it appears only after conversion into acetamide by β-oxidation.

Acetamide injected after fluoroacetate

As acetamide might interfere with the formation of fluorocitrate that takes place after the administration of fluoroacetate, the possibility of some protection being obtained by acetamide when given after the administration of fluoroacetate was reinvestigated. Previous negative results, it was argued, could have been due to the fact that the injection of acetamide was delayed at least 30 min. after administration of fluoroacetate; enough fluorocitrate might then have been formed in the cells to cause toxic effects. Further, it was known that after 30 min. a powerful inhibition of the tricarboxylic acid cycle had taken place, as indicated by the increased citrate levels in the tissue. However, some definite time must elapse after administration of fluoroacetate before a lethal concentration of fluorocitrate is reached, and acetamide given before the end of this period might still give protection by interfering with the formation of further fluorocitrate.

It was found that acetamide injected up to 8 min. after a dose of 10 mg. of sodium fluoroacetate/kg. protected white rats and kept them alive, whereas no control animal ever survived this dose.

DISCUSSION

Important progress towards the understanding of fluoroacetate poisoning was reached only after the disturbance of the tricarboxylic acid cycle, provoked by fluoroacetate, administered *in vivo* or added to kidney homogenates *in vitro* had been established (Liébecq & Peters, 1949; Buffa & Peters, 1949). The aberration found was a striking accumulation of citrate in different tissues as an immediate result of the treatment with fluoroacetate. The importance of this discovery could, however, be fully understood only in the light of the jamming hypothesis advanced by Liébecq & Peters (1949) and confirmed by subsequent work (Buffa & Peters, 1950; Potter *et al.* 1951; Peters & Wilson, 1952; Peters, Wakelin, Buffa & Thomas, 1953). An active tricarboxylic acid fraction containing fluorine was isolated from poisoned-kidney homogenates (Buffa, Peters & Wakelin, 1951), and the toxic compound is now thought to be fluorocitric acid (Peters *et al.* 1953), which inhibits aconitase (Lotspeich, Peters & Wilson, 1951), thereby jamming the tricarboxylic acid cycle.

The fact that acetamide and other substances reduce both the toxic effects and the citrate accumulation support the opinion of Peters (1952*b*) that it is the citrate accumulation which is partly or wholly responsible for the toxic effects of fluoroacetate.

Kandel, Johnson & Chenoweth (1951) and Kandel & Chenoweth (1952) doubted whether high citrate levels were related to the toxic effects of fluoroacetate administration, as they found that in

brain there was complete dissociation of citrate content and toxic symptoms. It may be questioned, however, whether the strange behaviour of brain necessarily indicates a negative conclusion as to the importance of the citrate accumulation. It seems more important to discover why fluoroacetate produces less citrate accumulation in brain and why brain reacts differently from other organs to acetamide.

Acetamide protected animals against fluoroacetate poisoning, without, however, being able to prevent the convulsions entirely. As acetamide gives protection when administered before a lethal accumulation of fluorocitrate has occurred as a result of fluoroacetate injection and does not give protection when enough fluorocitrate has been formed in the cell, it appears that acetamide is not capable of counteracting the fluorocitrate, but that it interferes with its formation. Fluorocitrate formation was dependent on the dose of fluoroacetate given, as measured by the citrate-accumulation effect; therefore it is understandable that a very high dose of fluoroacetate will be toxic even in the presence of acetamide, as in spite of the inhibition of fluorocitrate formation by acetamide a lethal concentration can still be reached. Also, acetamide given up to 8 min. after fluoroacetate inhibited further fluorocitrate formation and therefore had a protective action.

Both acetamide and monoacetin influence the reactions of the tricarboxylic acid cycle by causing increased citrate levels in the organs. In this connexion, Mallov & Tepperman (1953) have recently reported that ethanol also caused increased citrate levels in spleen and kidney, and Hutchens *et al.* (1949) obtained protection against fluoroacetate with ethanol.

The low toxicity of fluoroacetamide is also reflected by the increased citrate accumulation that it produces. The ratio of the values of LD_{50} for sodium fluoroacetate and fluoroacetamide is about 1:5; this is in accordance with the observation that a dose of 20 mg. of fluoroacetamide/kg. is needed to induce the same citrate accumulation as is caused by 5 mg. of sodium fluoroacetate/kg. The activity of fluoroacetamide may be due to the liberation of fluoroacetate by hydrolysis. It is not surprising that the hydrolysis should be slow in comparison with that of other fluoro compounds which act in the same way by transformation into fluoroacetate (Saunders & Stacey, 1948, 1949), because it is known from the work of Bray, James, Ryman & Thorpe (1948) and Bray, James, Thorpe, Wasdell & Wood (1949) that hydrolysis of acetamide *in vitro* by organ extracts is very slow.

The lower activity of fluoroacetamide is thus not of the same character as, for example, the non-toxicity of fluoropropionate or fluorosuccinate. These compounds are inactive, because they cannot participate in any condensation to yield fluorocitrate in which the C–F bond would become part of the fluoroacetyl portion of the fluorocitric acid molecule.

SUMMARY

1. The protective effect of acetamide in fluoroacetate poisoning is accompanied in heart and kidney by citrate concentrations that are significantly lower than those found without acetamide. Citrate accumulation in brain is not altered by acetamide.

2. Monoacetin also produces a decreased citrate level in fluoroacetate-treated animals. Both acetamide and monoacetin, however, produce increased citrate concentrations in brain, heart and kidney, when administered without fluoroacetate.

3. Citrate concentrations were proportional to the dose of fluoroacetate and to the period of time after administration of fluoroacetate.

4. Acetamide does not counteract the effect of fluoroacetate poisoning once this has led to a toxic concentration of fluorocitrate in the cells. It does, however, counteract the formation of fluorocitrate and gives protection to white rats when injected before and even up to 8 min. after administration of fluoroacetate.

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Studies of the Copper Compounds in Aqueous Extracts of Herbage

By C. F. MILLS
The Rowett Research Institute, Bucksburn, Aberdeenshire

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In a previous publication (Mills, 1955*a*) it has been shown that the physiological response of the copper-deficient rat may be used to compare the availability of copper in different dietary supplements. The results of this work indicated that the copper of an aqueous extract of freeze-dried herbage was more readily utilized for haemoglobin formation, melanogenesis, growth and liver-copper storage than an equivalent quantity of copper administered as cupric sulphate. The results of earlier work (Mills, 1954) suggested that much of the copper in herbage exists as organic complexes, but with the methods employed it was not possible to determine the contribution of ionic copper to the total copper content of the soluble components of herbage. The present investigation was designed to determine whether the water-soluble component of herbage which promotes rapid recovery of the copper-deficient rat contains copper mainly as ionic salts or as organic complexes which may or may not be broken down to liberate the free ion in the digestive tract.

EXPERIMENTAL

Electrodialysis of herbage extracts. A sample (20 g.) of milled, freeze-dried herbage was extracted with 200 ml. of glass-distilled water by stirring for 18 hr. at 37°. The mixture was filtered through muslin and the filtrate centrifuged at 8300 *g* for 1 hr. Before electrodialysis the supernatant was concentrated to 50 ml. by evaporation in a freeze drier. The apparatus described by Theorell & Åkeson (1943) was modified by substituting cellophan and filter-paper membranes for the parchment membranes used by these workers. This modification was necessary to prevent complex-formation between the Cu^{2+} ions of the dialysate and free amino and carboxyl groups of parchment protein. A further modification was the introduction of a fourth dialysis cell between the cell containing the sample and the catholyte cell (Fig. 1). Dialysis cells were constructed of $\frac{1}{8}$ in. Perspex and were connected by $\frac{1}{2}$ in. diam. holes in which were placed the membranes interposed between $\frac{1}{16}$ in. rubber gaskets. Electrodes were of 22 s.w.g. Pt wire wound in $\frac{1}{2}$ in. diam. spirals. Cooling water was circulated through glass U tubes in each cell and the cell contents were stirred with a stream of N_2. Power supplies were from a stabilized power unit of maximum output 100 mA at 250 v. Electrodialysis was carried out for about 9 hr., the current being restricted to 75 mA by a variable rheostat to avoid overheating the cell contents. After this period the current rapidly fell to about 3 mA, indicating that ion transport had virtually ceased. Determinations of pH on cell contents were carried out at intervals during electrodialysis. Cell contents were evaporated to dryness before Cu analysis.

Copper analyses. These were carried out by an adaptation of the method of Eden & Green (1940). The yellow Cu diethyldithiocarbamate complex was extracted into 2 ml. of amyl alcohol at 20° and the absorption at 432 mμ. was determined by a Beckman spectrophotometer.

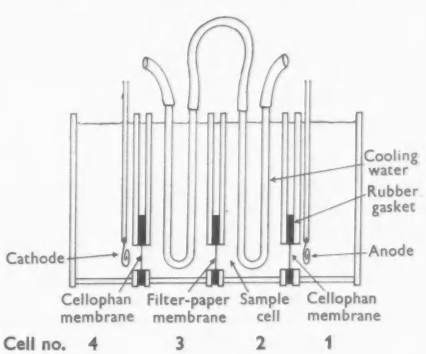

Fig. 1. Four-compartment electrodialysis unit.

Table 1. *Electrodialysis of a concentrated aqueous extract of freeze-dried herbage*

Time (min.)	Cell 1 (anode)	Cell 2 (sample cell)	Cell 3	Cell 4 (cathode)	Current (mA)
	(i) pH of cell contents during electrodialysis				
10	3·2	4·3	5·8	5·8	7
25	3·0	4·1	6·2	8·1	15
60	2·0	3·4	8·5	9·0	54
120	<2	3·2	8·0	9·5	75
480	<2	2·8	8·0	>10	4
	(ii) Migration of copper during electrodialysis				
Copper distribution after electrodialysis (μg. of Cu in cell)	0·20	6·92	0·19	0·26	—
	Total Cu in sample electrodialysed = 7·42 μg.				
	Total Cu recovered = 7·57 μg.				

Preparation and use of radioactive copper. Radioactive Cu was received in the form of irradiated Specpure foil. A sample (0·04 g.) was dissolved in 1 ml. of 70% (w/w) HNO_3 (redist. A.R.), and the excess of acid neutralized with 2N-NaOH (A.R.) with vigorous stirring to prevent precipitation of $Cu(OH)_2$. This solution was diluted and a portion containing 10 μg. (^{64}Cu + carrier Cu) was added to the freeze-dried concentrate corresponding to 150 ml. of the original aqueous extract of herbage. After dilution to 50 ml. the product was electrodialysed for 9 hr. After wet-ashing with nitric, sulphuric and perchloric acids the ^{64}Cu activity of cell contents was determined by means of a 10 ml. capacity Mullard liquid counter with Dynatron scaler unit.

Uptake of copper by a cation-exchange resin from herbage extracts after in vitro *peptic digestion.* Samples (5 ml.) of an aqueous extract of herbage prepared as described above were incubated for 3 hr. at 37° with 0·005 g. of pepsin (B.D.H.) at various pH values after the addition of 2N-HCl. After cooling to 18°, 2 g. (wet weight) of Zeo-Karb 215 (Na activated) resin was added to each digest and the pH adjusted to its original value by the addition of further acid. After being allowed to stand with frequent stirring for 1 hr. the supernatant liquor was decanted off and the resin washed with 30 ml. of glass-distilled water. The supernatant and resin washings were pooled for Cu analysis. To study the uptake of the free Cu^{2+} ion by Zeo-Karb 215 (Na) under similar conditions of pH, 1·85 μg. of Cu^{2+} as $CuSO_4,5H_2O$ (A.R.) was added to 5 ml. samples of the catholyte solution obtained by the electrodialysis of an aqueous extract of herbage; these solutions were then subjected to resin treatment as described above.

pH *determinations.* All pH determinations were carried out with the glass electrode.

RESULTS

Electrodialysis of aqueous extracts of herbage. The results of a typical electrodialysis of a concentrated aqueous extract of herbage are presented in Table 1. Only 0·26 μg. of the copper in the herbage preparation migrated through the toughened filter paper and cellophan membranes into the cathode compartment. A further 0·19 μg. of the copper migrated towards the cathode, but was prevented by the cellophan membrane from entering the cathode compartment. Other ions freely migrated during electrodialysis, as was shown by the changes in pH of the contents of cathode and anode compartments. Separate experiments have shown that the free Cu^{2+} ion in the absence of herbage extract readily migrates to the cathode, the migration of 50 μg. of copper being complete within 9 hr.

Table 2. *Partition of radioactive copper by electrodialysis of a herbage extract treated with* 10 μg. of ^{64}Cu

	Cell pH	^{64}Cu content of cell (μg.)
Cell 1 (anode)	<2	0·06
Cell 2 (sample cell)	3·1	9·85
Cell 3	7·5	0·03
Cell 4 (cathode)	>10	0·04

Recovery of added ^{64}Cu, 99·8%.

These results show that cationic copper is not readily liberated from the herbage extract, despite the low pH attained during the electrodialysis. Migration of copper into the anode compartment, presumably in the form of negatively charged complex ions, accounted for about 2·7% of the copper present in the original material. However, the substitution of the negatively charged cellophan membranes for the ampholytic parchment used by Theorell & Åkeson (1943) may prevent the efficient separation of complexes of weak negative charge from neutral materials. Thus it is probable that the material remaining in the sample cell after electrodialysis may contain some copper in the form of complexes of negative charge in addition to neutral complexes.

Effect of adding Cu^{2+} *ions to an aqueous extract of herbage.* The uptake of free Cu^{2+} ions by the water-soluble components of herbage was investigated by adding radioactive copper, as $^{64}Cu(NO_3)_2$, to a concentrated aqueous extract of herbage. The product was electrodialysed for 9 hr. The results are presented in Table 2. They demonstrate the avidity of the water-soluble components of herbage for ionic

copper. In the untreated herbage extract a considerable excess of free metal-binding ligands must be present, for, in this experiment, the copper content of the extract was more than doubled by the addition of the radioactive cupric ion, yet these ions were rapidly removed by complex formation and only 0·4% of the added copper migrated into the cathode cell.

Uptake of copper by Zeo-Karb 215 (Na) *from* in vitro *peptic digests of the water-soluble components of herbage.* In earlier work (Mills, 1955*b*) the uptake of copper by columns of the cation-exchange resins Zeo-Karb 215 and 226 (H^+-activated forms) from the water-soluble components of herbage was examined. It was found that under moderately acid conditions (pH range 2·6–4·9) only a very small proportion of the total copper was retained by ion exchange with either resin.

In the present investigation the uptake of copper by Zeo-Karb 215 (Na) under physiologically acid conditions after *in vitro* incubation of an aqueous extract of herbage was examined. The results are presented in Fig. 2. It is seen that above pH 2·5 less than 10% of the copper in the herbage extract was retained by the resin (curve *A*). Under more acid conditions copper uptake increased, until at pH 0·8 68% was retained. The gradual decline in copper uptake as the pH was further reduced is apparently the result of competition at ion-exchange sites by the other freely soluble cations of the herbage extract, as is seen on studying the uptake by the resin of Cu^{2+} ions added to the catholyte obtained from electrodialysis of a herbage extract. Thus above pH 1 more than 90% of the ionic copper is retained, but under more acid conditions the efficiency of the resin rapidly falls (curve *B*). In the absence of the other soluble cations of herbage, complete uptake of added Cu^{2+} ions is readily obtained even from N-HCl.

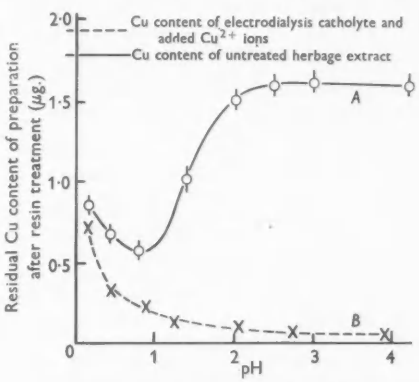

Fig. 2. Uptake of Cu by Zeo-Karb 215 (Na) from an aqueous extract of herbage incubated *in vitro* + pepsin and from an electrodialysis catholyte of herbage containing added Cu^{2+} ions as a function of pH. Curve *A*, ϕ—ϕ, residual Cu content of herbage extract after resin treatment (three determinations to each point: spread indicated by vertical line). Curve *B*, ×---×, residual Cu content of catholyte + Cu^{2+} ions after resin treatment.

DISCUSSION

The transport of copper to the cathode of an electrodialysis cell does not show unequivocally that this element is in the form of the free Cu^{2+} ion. Cathode-migrating species in electrodialysis include the free ion and positively charged stable complexes. Both these components would freely penetrate the filter-paper membrane on the cathode side of the sample cell in the electrodialysis unit. It is possible that positively charged complexes of large molecular size, although migrating in the direction of the cathode, may not be able to diffuse through the cellophan membrane and into the cathode compartment. Thus in this experiment about 5% of the total copper migrated towards the cathode, but the components migrating into the cathode compartment accounted for only 2·5% of the copper. From these results there is evidence to suggest that in an aqueous extract of herbage a small quantity of copper is present as the free ion or as positively charged complexes, but the greater part of the copper is in the form of neutral or negatively charged complexes which do not readily liberate the free ion under moderately acid conditions.

These suggestions are supported by the results of studies with Zeo-Karb 215 (Na). The soluble organic complexes of herbage appear to be stable above pH 2·5. Although a small proportion of the total Cu was retained by the cation-exchange resin above this pH, the retention appeared to be independent of pH; this suggests that the loss was incurred through adsorption of material in the resin matrix rather than through removal of copper by ion exchange.

SUMMARY

1. Electrodialysis of aqueous extracts of freeze-dried herbage in a modified Theorell–Åkeson four-compartment cell liberated only a small proportion of the copper in the extracts as the free ion. The greater part of the copper was present in the form of neutral or anionic complexes.

2. The avidity of the complex-forming ligands of an aqueous extract of herbage for added Cu^{2+} ions was demonstrated by adding radioactive copper to the extract before electrodialysis.

3. The organic complexes of copper in these extracts appeared to be stable in the presence of a cation-exchange resin after *in vitro* incubation at pH values above 2·5. Below pH 2·0 decomposition of complexes was appreciable.

I wish to thank Dr J. Duckworth for his interest and encouragement and Dr R. L. M. Synge for advice on electrodialysis techniques. Thanks are due to Miss Grace Murray and Mr A. Forbes for skilled technical assistance.

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The Dietary Availability of Copper in the Form of Naturally Occurring Organic Complexes

By C. F. MILLS
The Rowett Research Institute, Bucksburn, Aberdeenshire

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In the preceding paper (Mills, 1956) it was shown that the greater part of the copper in an aqueous extract of herbage was in the form of complexes which appear to be stable above pH 2·5. Earlier it had been found that such extracts promoted a more rapid recovery of the copper-deficient rat than a corresponding quantity of copper administered as an inorganic supplement (Mills, 1955).

The object of the present work was to investigate the availability of copper in herbage extracts treated with a cation-exchange resin to remove traces of ionic copper and copper from complexes of low stability and to determine whether the rapid response to such preparations in earlier biological trials was due to either: (1) the efficient utilization of any ionic copper present in the original extract or liberated from complexes during gastric digestion, or (2) the utilization of stable organic complexes of copper.

The use of the copper-deficient rat to determine the biological availability of dietary copper has been described elsewhere (Mills, 1955). The technique consists in depleting the weanling black-hooded rat of liver-copper reserves on a copper-deficient basal diet supplemented with vitamins and with a purified-iron salt. As the animal becomes copper-deficient a marked anaemia develops, with severe depigmentation of hair and failure of growth. The effect of a dietary supplement containing copper on haematopoiesis, melanin-pigment regeneration, growth and liver-copper storage is taken as an index of the biological availability of copper in the test material.

EXPERIMENTAL

Preparation of 'de-ionized' herbage extract. [The term 'de-ionized' herbage extract is in this paper used to indicate a herbage extract treated with Zeo-Karb 215 (H) to remove ionic copper.] Milled, freeze-dried herbage (200 g.) was stirred vigorously for 18 hr. with 2 l. of glass-distilled water at 37°. Coarse solid material was filtered off with muslin and the filtrate centrifuged at 1500 *g* for 1 hr. at −0°. A 300 mm. × 18 mm. column of Zeo-Karb 215 was activated with 4 vol. of 2N-HCl (A.R.) and washed with glass-distilled water until the eluate was chloride-free. The centrifuged herbage extract was percolated through the cation-exchange resin at the rate of 5 ml./minute. The first samples of eluate were strongly acid (pH < 1), but after about 45 ml. of eluate had been run off the pH rose rapidly to 3·4 and the collection of a bulk sample was commenced. This extract was freeze-dried and then reconstituted with glass-distilled water so that 0·5 ml. of concentrate contained 3·0 μg. of Cu. This concentrate was stored under N_2 at +1°.

Production of copper-deficient rats. Rats of the Rowett Institute strain of black-hooded Lister were used for this work. (i) Pre-weaning treatment: fresh whole milk was gathered into acid-washed glass vessels and was supplemented with the following vitamins (mg./l.) before feeding *ad lib.* to dam and litter: thiamine 0·3, riboflavin 0·4, Ca pantothenate 2·0, folic acid 0·2, nicotinic acid 0·2, *p*-aminobenzoic acid 0·2 (added in aqueous solution); vitamin A (alcohol) 0·8, α-tocopherol acetate 5·0 (added in arachis oil). This supplemented milk was shaken vigorously before use to ensure uniform dispersion of arachis oil containing fat-soluble vitamins. The dam was removed to a separate cage for three 1 hr. periods daily for the feeding of a solid diet. (ii) Post-weaning depletion diet: a solid basal diet of the following composition was fed at the rate of 6 g./rat/day for the first two weeks after weaning and 10 g./rat/day thereafter: roller-dried full-cream milk (low Cu batch) 95%, sucrose (A.R.) 5%. To this the following water-soluble vitamins (mg./kg.) were added: thiamine 5·0, riboflavin 10·0, pyridoxine 5·0, nicotinic acid 20·0, Ca pantothenate 50·0, *p*-aminobenzoic acid 10·0, folic acid 11·0, inositol 400·0. The copper content of this diet was 0·18 μg./g. of D.M. A purified $FeCl_3$ supplement was administered orally to give 0·5 mg. of Fe^{3+}/rat/day. The following fat-soluble vitamins were administered orally in 0·2 ml. of arachis oil: vitamin A (alcohol), 37 μg./rat/day;

α-tocopherol acetate, 2·4 mg./rat/day. A marked copper-deficiency anaemia developed after feeding this diet for 6 weeks, whereupon animals were randomized into experimental groups (see Table 2).

pH *determinations on stomach contents.* Animals receiving the diet described above were killed by decapitation. The stomach was removed under liquid paraffin and a glass-electrode system inserted through an incision in the stomach wall. All readings were obtained within 1 min. of death. Results are presented in Table 1.

Haemoglobin determinations. Blood samples were obtained by puncture of the coccygeal artery and vein. Haemoglobin was determined by a modification of the method of Nicholas (1951), the optical density of oxyhaemoglobin being measured at 541 mμ. in a Beckman spectrophotometer.

Hair-pigment assessment. A series of pelts were prepared from black-hooded rats receiving different levels of dietary copper. The pelts ranged in colour from black to pale silver-grey. Experimental animals were given a 'pigmentation index' by comparison with these pelts, an index of 10 indicating a fully pigmented head and an index of 1 a very severely depigmented animal.

Uptake of ^{64}Cu by the rat liver. A sample (0·04 g.) of irradiated Specpure Cu foil was dissolved in 1 ml. of 70% (w/w) HNO_3 (A.R.) and the excess of acid neutralized with 2N-NaOH while being stirred vigorously to prevent precipitation of $Cu(OH)_2$. The uptake of ^{64}Cu by the rat liver was compared after feeding 10 μg. of the irradiated Cu [10 μg. of (^{64}Cu + inert ^{63}Cu) as received from A.E.R.E. Harwell] (i) as the free Cu^{2+} ion, and (ii) after combining the irradiated Cu with a reconstituted freeze-dried aqueous extract of 20 g. of herbage. (The product was freed from traces of the free $^{64}Cu^{2+}$ ion by passage through a H^+-activated Zeo-Karb 215 column at pH 3·4 as described above.) Animals were depleted of copper reserves before this experiment, using the basal diet, vitamin and iron supplements described above. Food was withdrawn 3 hr. before feeding radioactive supplements. Animals were killed by coal gas. Livers were removed, washed in distilled water and prepared for liquid counting by the technique of Comar, Davis & Singer (1948). Samples were counted in a 10 ml. capacity Mullard ethanol-quenched liquid counter.

RESULTS

Biological availability trial. The physiological response obtained from the feeding of 3 μg. of copper/day as the stable copper complexes of a 'de-ionized' herbage extract was compared with that obtained from 3, 6, 10 and 15 μg. of the Cu^{2+} ion administered as cupric sulphate. The results are presented in Table 2. The group-mean haemoglobin levels at intervals throughout the trial and the standard deviations of these values are presented graphically in Fig. 1. There were significant differences in final haemoglobin levels between treatments ($P < 0.001$). The results fell into two groups with treatments 1 and 2 (3 μg. of Cu^{2+} and

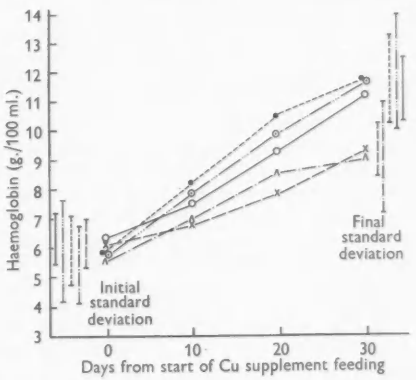

Fig. 1. Haemoglobin recovery curves of copper-deficient rats fed on copper supplements (mean of results for group): ×——×, 3 μg. Cu^{2+}/day (6 animals); Λ—·—Λ, 6 μg. Cu^{2+}/day (6 animals); ●- - -●, 10 μg. Cu^{2+}/day (5 animals); ⊙—···—⊙, 15 μg. Cu^{2+}/day (8 animals); ○—○, 3 μg. copper/day in 'de-ionized' herbage extract (6 animals).

Table 1. pH *of stomach contents of rats fed on copper-deficient basal diet*

Time after feeding (hr.)	2	6	10	17
Range of pH values of stomach contents	4·3–4·0	3·8–3·5	3·8–2·8	3·6–3·4
Mean	4·1	3·7	3·3	3·5
(No. of animals)	(4)	(4)	(4)	(3)

Table 2. *Group mean values and standard errors for haemoglobin level, weight gain, liver copper and change in pigmentation index of rats fed on ionic copper or a de-ionized extract of herbage for 30 days*

Treatment	Final haemoglobin level (g./100 ml.)	Wt. gain during period (g.)	Total liver Cu (μg.)	Change in pigmentation index during period	No. of animals receiving treatment
(1) 3 μg. Cu^{2+}/day as $CuSO_4,5H_2O$ (A.R.)	9·4±0·6	19±4	9·7±0·8	+1	6
(2) 6 μg. Cu^{2+}/day as $CuSO_4,5H_2O$ (A.R.)	9·2±0·6	25±4	10·9±0·8	+1	6
(3) 10 μg. Cu^{2+}/day as $CuSO_4,5H_2O$ (A.R.)	11·8±0·6	30±4	12·8±0·9	+2	5
(4) 15 μg. Cu^{2+}/day as $CuSO_4,5H_2O$ (A.R.)	11·8±0·5	35±4	13·0±0·7	+2	8
(5) 3 μg. Cu/day as 'de-ionized' herbage extract	11·1±0·6	30±4	13·2±0·9	+2	6

Table 3. *Uptake of* ^{64}Cu *by the rat liver when fed in combination with various supplements*

Group treatment	Cu recovered in liver (% of administered dose)	No. of animals	Mean log* of liver copper
Expt. 1 (period 24 hr.)			
(i) 10 μg. ^{64}Cu + carrier Cu fed as free ion + 2 g. sucrose	1·4	5	1·13
(ii) 10 μg. ^{64}Cu + carrier Cu combined with aqueous extra of herbage fed + 2 g. sucrose	4·0	5	1·58
Standard error of difference of mean logs* = $\pm$0·09 (8 degrees of freedom).			
Expt. 2 (period 36 hr.)			
(i) 10 μg. ^{64}Cu + carrier Cu fed as free ion + 2 g. sucrose	3·0	5	1·47
(ii) 10 μg. ^{64}Cu + carrier Cu mixed with 5 g. dried milk basal diet + 2 g. sucrose before feeding	7·0	5	1·84
(iii) 10 μg. ^{64}Cu + carrier Cu combined with aqueous extract of herbage fed + 2 g. sucrose	9·4	5	1·96
Standard error of difference of mean logs* = $\pm$0·06 (12 degrees of freedom).			

* To stabilize the variance, the log of liver-copper values were analysed rather than the values themselves.

6 μg. of Cu^{2+}/rat/day) giving lower final haemoglobin levels than treatments 3, 4 and 5 (10 μg. of Cu^{2+}, 15 μg. of Cu^{2+} and 3 μg. of Cu in 'de-ionized' herbage extract/rat/day). Neither the differences in weight gain or in liver-copper storage at the end of the 30 day experimental period were quite large enough to reach significance, but in each case the mean values obtained suggested a pattern similar to that of the haemoglobin results with the 3 μg. of Cu^{2+} and 6 μg. of Cu^{2+}/day treatments giving lower values than the other three treatments. This pattern of differences was repeated in the results for hair-pigment regeneration.

These results demonstrate that the 'de-ionized' herbage extract contains a source of copper which is readily available to the copper-deficient rat. The rate of recovery of the animals receiving 3 μg. of copper daily in this product was superior in all measures to that obtained by feeding 3 μg. of copper as cupric sulphate. The response to the feeding of herbage extract was approximately equivalent to that obtained with 10 μg./day of the free Cu^{2+} ion.

Uptake of ^{64}Cu *by the rat liver.* As shown in Table 3 (Expt. 1) approximately 2·8 times as much radioactive copper was absorbed and stored in the rat liver when fed in combination with the metal complex-forming ligands of an aqueous extract of herbage as when fed as the free Cu^{2+} ion. This difference was highly significant with $P < 0{\cdot}005$. In this, as in all previous copper-availability trials with the rat, an attempt was made to minimize interactions between copper supplements and the constituents of the basal diet by feeding these materials at different times. In a separate experiment, the results of which are presented in Table 3 (Expt. 2), the effect of mixing the radioactive Cu^{2+} ion with the dried-milk basal diet was investigated. As in the previous experiment more copper was taken up by the liver when combined with a herbage extract than when fed as the free ion ($P < 0{\cdot}001$). Although an increased uptake was obtained by allowing the free ion to combine with the milk basal diet before feeding, the results suggest that copper fed in this form is still not as efficiently utilized as that fed in combination with the herbage extract ($0{\cdot}05 < P < 0{\cdot}1$).

DISCUSSION

The results of pH determinations on the stomach contents of rats receiving the diet used in these experiments indicate that pH fluctuations in the range 4·3–2·8 might be expected. It has been shown (Mills, 1956) that only below pH 2·5–2·0 are appreciable quantities of ionic copper liberated from the copper complexes of an aqueous extract of herbage during *in vitro* incubation. To account for the observation that the copper of such extracts is readily utilized by the rat it is necessary to consider the possible fate of orally ingested copper in the digestive tract.

Albert (1951) has shown that the Cu^{2+} ion may combine readily with many biological materials to form complexes, and Albert & Gledhill (1947) and McNaught, Owen & Smith (1950) have shown that such complexes may form under physiological conditions. The solubility and stability of such complexes may vary greatly. Thus complex-formation with most amino and hydroxy acids results in soluble products, whereas the copper complexes formed with some proteins, with

porphyrins and with lignin are insoluble at normal physiological pH.

Evidence is however available to suggest that certain soluble complexes of copper may be more readily transported through biological membranes than the free ion. Thus Uzman (1953) has demonstrated that the urinary excretion of copper in hepatolenticular degeneration is associated with the excretion of specific copper oligopeptide complexes. The mobilization and renal excretion of body copper as the Cu–BAL complex has been demonstrated by Denny-Brown & Porter (1951). Saha & Guha (1941) found that an iron–copper nucleoprotein complex isolated from fish muscle promoted a more rapid regeneration of haemoglobin than equivalent quantities of ionic iron and copper fed to the copper- and iron-deficient rat. This complex was readily absorbed through the small intestine mucosa of the rabbit (Saha & Bannerjee, 1943). Seelemann & Baudissin (1954) obtained a greater stimulation of haematopoiesis and growth in the rabbit from the sodium salt of a copper allylthiourea–benzoic acid complex than from inorganic copper.

From the foregoing it appears possible that, in the present work, the rapid recovery of the copper-deficient rat fed on an aqueous extract of herbage treated with a cation-exchange resin may be due, in part, to the presence of soluble complexes which are stable at the pH encountered in the stomach and which are readily absorbed from the jejunum (the site of copper absorption according to Sachs, Levine, Hill & Hughes, 1943).

It is suggested that the transport of ionic copper through the intestinal mucosa may not be the only mechanism whereby this element is absorbed. If, as the results described above appear to indicate, copper may be absorbed as stable, soluble complexes, it is possible that such complexes could arrive at the site of absorption from two sources: (1) from specific complexes preformed in a biological food material (e.g. herbage); or (2) from the interaction of ingested Cu^{2+} ions with specific complex-forming ligands in the digestive-tract contents. It would be expected that since, in this last case, the free ion may alternatively combine with other constituents to form insoluble products or complexes not suitable for absorption, the utilization of copper in this form would be poor. This suggestion is supported by results in the present study and by Coulson, Remington & Lynch (1934), Comar *et al.* (1948) and Schubert, Maurer & Riezler (1948) who have all demonstrated the poor utilization of the orally administered Cu^{2+} ion.

SUMMARY

1. Copper-deficient black-hooded rats were used to study the availability of copper in 'deionized' aqueous extracts of herbage. Copper uptake by the rat was assessed by haematopoietic response, melanin-pigment regeneration, growth and measurement of liver-copper storage.

2. Aqueous extracts of herbage passed through a cation-exchange resin to remove the Cu^{2+} ion still contained copper in an available form. The stable, soluble complexes of copper remaining in these extracts promoted a more rapid physiological response and a greater storage of copper in the liver than the feeding of equivalent amounts of the Cu^{2+} ion.

3. These results were confirmed by comparing the uptake of ^{64}Cu by the rat liver after feeding the isotope as the $^{64}Cu^{2+}$ ion and after combining it with the complex-forming ligands of an aqueous herbage extract.

4. To account for the rapid utilization of the copper of the stable, soluble complexes of herbage it is suggested that copper may be transported through the intestinal mucosa in the form of such complexes.

I wish to thank Dr J. Duckworth for many helpful discussions during this work. Thanks are due to Mr A. W. Boyne for the statistical analysis of results. I am indebted to Miss Grace Murray for skilled technical assistance and to Messrs J. W. C. Davis and N. Robertson for help with experimental animals.

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Studies on the Concentration of Radioiodide and Thiocyanate by Slices of the Salivary Gland

By K. FLETCHER, A. J. HONOUR AND E. N. ROWLANDS
Medical Research Council Department of Clinical Research, University College Hospital Medical School, London

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Human salivary glands concentrate iodide to an extent comparable with that of the iodide trap of the thyroid gland (Honour, Myant & Rowlands, 1952), and there is a very close analogy between the effects of antithyroid drugs on the concentration of iodide by the thyroid and salivary glands (Rowlands, Edwards & Honour, 1953). Thus it seemed likely that a study of the concentration of iodide by salivary glands might help to elucidate the mechanism of the iodide trap of the thyroid, which is much more difficult to study as it is only the first stage in the synthesis of thyroid hormone, whereas organic binding of iodide does not occur in salivary glands.

Edwards, Fletcher & Rowlands (1954) found evidence of antagonism between iodide, perchlorate, thiocyanate and nitrate for secretion in human saliva, the most likely mechanism being that they compete as substrates for receptors on a protein which transports them across the salivary cells. To study the mechanism in more detail it was necessary to obtain preparations of salivary gland which could be studied *in vitro*. Although the concentration of iodide in the saliva of both man and the guinea pig is about 30 times the plasma level, the concentration in their salivary glands is the same as, or only slightly greater than, the plasma iodide; the rat does not concentrate iodide in its saliva or salivary glands. We therefore used the mouse as an experimental animal, following Collins & Hellmann's (1955) observation that it concentrates iodide in its salivary glands, an observation which we have confirmed. This paper describes the effect of various factors on the concentration of [^{131}I]-iodide by slices of the salivary gland of the mouse.

METHODS

Paper chromatography and autoradiography. The procedures described below were adopted to exclude the possibility that synthesis of organic iodine was occurring in the salivary gland. A mouse was given 20 μc of ^{131}I as iodide by intraperitoneal injection. One hour later, it was killed and its salivary glands were removed. These were ground with sand and the resulting breis were completely extracted with *n*-butanol. After evaporation, samples of the butanol extract were applied to chromatography paper and developed either in *n*-butanol–dioxan–ammonia (Gross, Leblond, Franklin & Quastel, 1950), or in *n*-butanol–2N acetic acid. A similar technique was used to obtain chromatograms from slices of salivary glands which had been incubated for 40 min. in an oxygenated saline solution containing $^{131}I^-$ at 37°. Autoradiography of the dried chromatograms obtained by both methods showed all the activity to have migrated as a single spot corresponding to added carrier iodide; no other compounds were detected.

Preparation and distribution of tissue slices. In vivo, the concentration of iodide in the salivary glands of a mouse weighing approx. 35 g. is about eight times the plasma level, but this ratio increases up to 15 with increasing weight of the animal. Male albino mice (Tuck's no. 1 strain, The Mousery, Rayleigh, Essex.) weighing 40–55 g. were therefore used in these experiments. *In vitro*, it was found that slices of salivary glands from different mice varied in their power of concentrating $^{131}I^-$ from the surrounding medium, the concentration ratio varying from 5 to 10. As several mice were used in each experiment, the following technique was devised to eliminate the effects of this variation as far as possible. Three mice were killed and the submaxillary glands dissected out as rapidly as possible. Each gland was sliced into three pieces by the method of Deutsch (1936), and each of these pieces was cut in half and placed in dishes containing buffered-saline solution (adjusted to pH 7·4 by the addition of M-H_3PO_4) of the following composition: water 100 ml., NaCl 850 mg., KCl 42 mg., $CaCl_2$ 24 mg., glucose 100 mg., Na_2HPO_4 60 mg. The pieces were then gently blotted on filter paper, weighed on a torsion balance and then transferred to six Warburg flasks in such a way that each flask contained a portion of tissue from each gland, the average weight of tissue in each flask being approximately 120 mg.

Incubation of slices and estimation of $^{131}I^-$ *concentration.* Two of the six flasks in each experiment contained 3·5 ml. of saline and served as controls. The other four contained different concentrations of the substance under test, dissolved in saline solution to a final volume of 3·5 ml., except when dinitrophenol and triiodothyroacetic acid [3:5-diiodo-4-(4-hydroxy-3-iodophenoxy)phenylacetic acid] were used, these being placed in the side arm. Carrier-free ^{131}I, as iodide, dissolved in 0·5 ml. of the saline medium, was placed in the side arm of each flask. The flasks were gassed with O_2 (100%) for 5 min. and then placed in the bath at 37°. After equilibration at bath temp. (10 min.) the flasks were sealed and the $^{131}I^-$ was tipped into the main compartment. The slices were incubated for about 40 min. and O_2 consumption was measured in the conventional manner. This period of time was chosen because the concentration of $^{131}I^-$ in the tissue did not increase with longer periods of

incubation. After incubation, the tissues were removed from the flasks and blotted, and digested in 2N-NaOH. Samples of the saline medium were also taken from each flask, and after adjustment of volume the radioactivity of both tissues and fluid was determined in an M 6 liquid counter.

From the known weight of tissue in each flask the ratio of the concentrations of $^{131}I^-$ in tissue (T) and medium (M) was derived:

$$T/M \text{ for } ^{131}I = \frac{\text{counts/g. of tissue}}{\text{counts/ml. of medium}}.$$

Estimation of thiocyanate. The tissues were distributed and incubated as described above, except that ^{131}I was not added, and after incubation the slices were removed from the flasks and homogenized in a small, all-glass, Potter–Elvehjem homogenizer in a known volume of water. The homogenate (0·5 ml.) was treated with 0·3 ml. of 80% (w/v) trichloroacetic acid and centrifuged, and thiocyanate was determined on the supernatant by the method of Aldridge (1945). Thiocyanate estimations were also made on the saline medium in each flask.

RESULTS

The chromatographic studies showed that synthesis of organic iodine did not occur in the salivary glands during the period of these experiments. Concentrations of methylthiouracil varying from $4{\cdot}4 \times 10^{-5}$ to $1{\cdot}3 \times 10^{-3}$M had no effect on the uptake of $^{131}I^-$ by the slices.

Effect of carrier-iodide $^{127}I^-$ and bromide on the concentration ratio (T/M) for $^{131}I^-$

When $^{127}I^-$ is added to the suspending medium in varying concentrations, the absolute amount of iodide in tissue and medium can be estimated by measuring the radioactivity in each, since the amount of iodide initially present in the gland is negligible. Fig. 1 shows the amount of iodide in the tissue at different concentrations in the medium; the concentration in the tissue rises with increasing concentrations in the medium but the concentration ratio (T/M) falls. However, even when large amounts of $^{127}I^-$ are added to the medium, the concentration ratio does not fall below about 1·0; indeed, it has not been possible to depress the ratio below 0·8, either by adding very high concentrations of powerful inhibitors to the suspending medium or even by boiling the gland after slicing it.

In Fig. 1 there is a considerable scatter of the experimental points about the curve because they represent the results of three separate experiments. In any one experiment, the slices are so distributed that differences in the concentrating power of the glands from different mice are reduced, but these differences may be considerable between one experiment and another. If, however, for each point on the graph the amount of iodide in the tissue is divided by the concentration ratio (T/M) of the corresponding control, the scatter is greatly diminished. In effect this expresses the amount of iodide in the gland per unit of concentrating power rather than per unit weight of tissue. In this way a straight-line relationship is obtained as shown in Fig. 2.

Potassium bromide in concentrations of 1×10^{-3} to 1×10^{-5}M had no effect on the concentration ratio (T/M) for $^{131}I^-$.

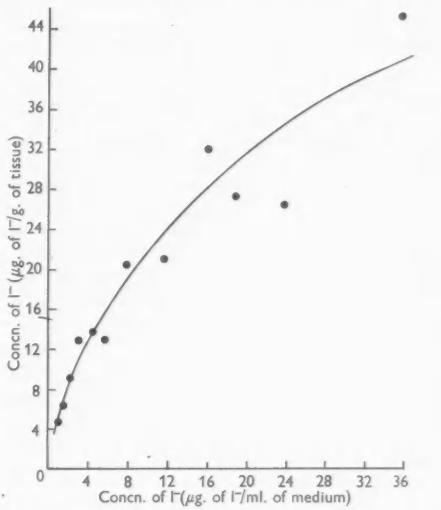

Fig. 1. Relationship between concentration of I^- in tissue and in medium.

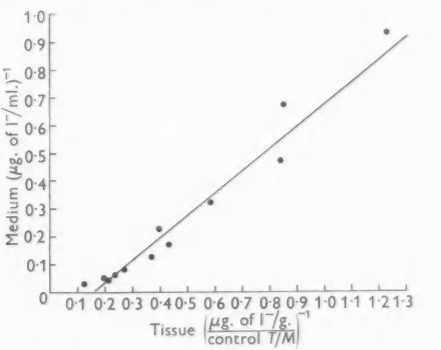

Fig. 2. Relationship between the reciprocal of the concentration of iodide/g. of tissue divided by the concentration ratio (T/M) of the control, and the reciprocal of the amount of iodide/ml. of medium.

Effect of ClO_4^-, SCN^-, *and* NO_3^- *on the concentration ratio* (T/M) *for* $^{131}I^-$

The potassium salts of each of these three anions depressed the uptake of $^{131}I^-$ when added in suitable concentration to the suspending medium. The range of concentrations used was 5×10^{-7} to $3{\cdot}8 \times 10^{-5}$ M of ClO_4^-, $2{\cdot}1 \times 10^{-5}$ to $4{\cdot}5 \times 10^{-4}$ M of SCN^-, and 2×10^{-4} to 4×10^{-3} M of NO_3^-. Perchlorate was 10 times as effective per equivalent as thiocyanate, and 80 times more effective per equivalent than nitrate, in lowering the concentration ratio (T/M). Fig. 3 shows the relative effects of ClO_4^- and SCN^- as inhibitors, and the effect of added $^{127}I^-$ is also plotted for comparison. The perchlorate ion is a very powerful inhibitor, since a concentration as low as 5×10^{-1} μM has a significant effect, and a concentration of 60–80 μM usually lowers the concentration ratio (T/M) to about 0·8. The ratio remains at about 0·8 even when enormous concentrations of the order of 4 mM are used.

Table 1. *Observed and calculated effect on the concentration ratio* (T/M) *for* $^{131}I^-$ *of adding* ClO_4^- *and* SCN^- *simultaneously to the medium*

T, $^{131}I^-$ counts/g. of tissue; M, $^{131}I^-$ counts/ml. of medium.

Concn. of ClO_4^- (μM)	Concn. of SCN^- (μM)	Observed T/M for $^{131}I^-$	Calculated T/M for $^{131}I^-$
5·06	345	14·5	17·4
15·1	129	14·7	16·7
7·54	43·1	19·0	24·2
5·02	108	20·4	23·7
2·51	134	22·1	25·2
1·76	32·4	36·2	39·5
0·754	32·3	41·9	46·0
1·76	10·8	43·2	48·0

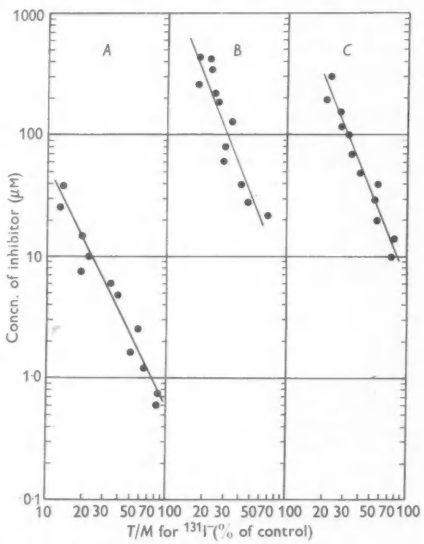

Fig. 3. Effect of ClO_4^- (*A*), SCN^- (*B*), and I^- (*C*) on the concentration ratio of $^{131}I^-$.

The similarity of the curves in Fig. 3 suggests that the mechanism of inhibition of iodide uptake may be similar for all three anions. Perchlorate and thiocyanate were therefore added simultaneously to the incubating medium to determine whether a summation of their individual effects occurred (Table 1). The third column in the table shows the observed concentration ratio (T/M) expressed as a percentage of the controls for each mixture of different concentrations of the two anions. Column 4 shows the calculated concentration ratios, assuming that the effect of the mixture represents a summation of the effect produced by either anion separately. The calculated concentration ratios were found in the following manner. The expected inhibitory effect of the ClO_4^- in each mixture was first read off from the curve for ClO_4^- in Fig. 3; then from the curve for SCN^- the amount of SCN^- necessary to produce this inhibitory effect was determined. The sum of the amount thus determined and the amount of SCN^- added to the mixture was found, and the inhibition by this total was read off from the SCN^- curve. The similarity of the observed and calculated results suggests a summation of the effects of these two anions.

Effect of endogenous SCN^- *on the concentration ratio* (T/M) *for* $^{131}I^-$

Table 2 shows the concentration of SCN^- in the tissue when different amounts of SCN^- are added to the suspending medium. It is evident that very large amounts have to be added to the medium to raise the concentration in the tissue significantly. Thus a concentration of SCN^- of $8{\cdot}6 \times 10^{-2}$ mM in the medium did not increase the concentration in the gland, yet this amount of added SCN^- depresses the concentration ratio (T/M) for $^{131}I^-$ by about 70 %.

The concentration of SCN^- present in the plasma of the mouse is 2·83 μg./ml. (48·8 μM; mean of six determinations, range 2·18–3·68). The concentration *in vivo* in the submaxillary salivary gland is 28·1 μg./g. (mean of three determinations, range 26·9–28·8). Thus the concentration ratio *in vivo* for SCN^- is about 10. It will be seen that the amount of SCN^- present in the gland is large compared with that which has to be added in order to depress T/M for $^{131}I^-$. Furthermore, from the data of Fig. 3 the amount of SCN^- in the plasma, 2·83 μg./ml. (48·8 μM), should cause considerable depression of iodide concentration.

Table 2. *Effect of the addition of* SCN^- *to the medium on the amount of* SCN^- *in the tissue and on the concentration ratio* (T/M) *for* SCN^-

Concn. of SCN^- in medium (μg./ml.)	Concn. of SCN^- in tissue (μg./g.)	Concn. ratio for SCN^- (T/M)
0·8	27·1	34·8
0·9	25·6	27·7
0·9	34·4	36·6
1·1	26·1	24·6
1·1	27·3	25·1
1·3	27·9	21·4
1·8	28·9	16·3
3·3	27·4	8·3
3·5	31·0	8·9
5·2	36·1	6·9
6·9	37·4	5·4
9·7	27·2	2·8
12·9	36·2	2·8
28·0	43·3	1·5
49·0	56·7	1·2

Table 3. *Comparison of the concentration ratio* (T/M) *for* $^{131}I^-$ *before and after leaching out* SCN^- *from the tissue*

Conc. of SCN^- in tissue (μg./g.)	T/M for $^{131}I^-$	Conc. of SCN^- in tissue after leaching (μg./g.)	T/M for $^{131}I^-$ after leaching
28·50	5·06	19·9*	6·74
30·91	8·54	16·5†	7·26
22·90	7·75	8·0†	6·43

* Leached for 30 min. in saline.
† Leached for 60 min. in saline.

The following observations were therefore made on tissue slices *in vitro* to study the effect of the high concentration of endogenous SCN^- in the glands on the uptake of $^{131}I^-$. It seemed possible that the concentration ratio for $^{131}I^-$ would rise if the endogenous SCN^- could be leached out of the tissue. Slices of gland were incubated in 20 ml. of saline solution for varying periods of time, and the amount of SCN^- remaining in the tissue was estimated (Table 3). Even after an hour's incubation, however, approximately 40% of the SCN^- was still present in the tissue, whereas only 10% should still be present under these experimental conditions to maintain the usual concentration ratio of about 30 between tissue and medium. In contrast to this it was found that $^{131}I^-$ could be leached out of a gland very rapidly. A mouse was injected with a dose of $^{131}I^-$ and killed an hour later, and its salivary glands were dissected out, sliced and immersed in 20 ml. of saline. Within 30 min. the usual concentration gradient between tissue and medium had been reached. Table 3 also shows the effect of incubating slices for 30 min. in saline to leach out some of the SCN^-, and then transferring them to Warburg flasks and measuring their uptake of $^{131}I^-$; the concentration ratio (T/M) for $^{131}I^-$ did not differ significantly from that of the controls in which the slices had not been previously immersed in saline.

Experiments were then carried out to determine whether the concentration of SCN^- in the slices could be lowered by adding either I^- or ClO_4^- to the medium. Table 4 shows that these anions had no effect on the endogenous SCN^- even in doses far exceeding those required to depress the concentration ratio for $^{131}I^-$ to 0·8.

Effect of dinitrophenol, triiodothyroacetic acid and thyrotrophic hormone

ClO_4^-, SCN^-, NO_3^- and $^{127}I^-$ depress the uptake of $^{131}I^-$ by the tissue without affecting its O_2 consumption significantly. On the other hand, 2:4-dinitrophenol in concentrations of 10^{-3} to 10^{-5} M depressed both O_2 consumption and T/M for $^{131}I^-$ (Table 5).

In view of the action of dinitrophenol on the tissue slices we tried the effect of adding triiodothyroacetic acid to the suspending medium, as it seemed possible from the results of Thibault & Pitt-Rivers (1955) that an increase in both O_2 consumption and uptake by the tissues of $^{131}I^-$

Table 4. *Effect on concentration of* SCN^- *in the tissue of adding* (1) 300 μM $^{127}I^-$, *and* (2) 100 μM ClO_4^- *to the medium*

Concn. of SCN^- in tissue (μg./g.)	Concn. of $^{127}I^-$ in medium (μM)	Concn. of ClO_4^- in medium (μM)
Expt. 1		
22·7	—	—
24·5	—	—
23·9	300	—
26·8	300	—
Expt. 2		
31·3	—	—
36·3	—	—
33·4	—	100
28·3	—	100

Table 5. *Effect of the addition of* 2:4-*dinitrophenol to the medium on the concentration ratio* (T/M) *for* $^{131}I^-$ *and on* q_{O_2}

Concn. of 2:4-dinitrophenol in medium (M)	T/M for $^{131}I^-$	q_{O_2} (μl. of O_2/mg. wet wt./hr.)
0	7·84	2·25
0	7·09	1·69
10^{-5}	5·56	1·88
5×10^{-5}	2·69	1·26
10^{-4}	2·18	1·12
10^{-3}	2·31	0·79

Table 6. *Action of various concentrations of triiodothyroacetic acid on the concentration ratio (T/M) for $^{131}I^-$ and oxygen consumption in salivary glands*

	Control		Triiodothyroacetic acid		
Concn. (μM)	T/M for $^{131}I^-$	q_{O_2} (μl. of O_2/mg. wet wt./hr.)	T/M for $^{131}I^-$	q_{O_2} (μl. of O_2/mg. wet wt./hr.)	Incubation times (min.)
1·0	3·94	2·03	5·46	2·26	20
			6·74	1·89	20
1·0	2·67	1·56	2·70	1·83	15
	2·81	1·82	2·60	1·85	15
			2·81	1·86	15
			2·37	2·13	15
1·0	3·64	1·99	2·32	0·79	20
	3·23	1·87	3·62	2·08	20
			3·93	1·90	20
			4·01	1·84	20
1·0	8·73	1·92	9·34	2·07	40
			9·94	1·90	40
1·61	2·18	1·82	3·49	1·91	15
			3·13	1·74	15
1·61	3·85	1·92	4·96	1·92	25
			3·74	1·78	25
1·61	2·51	1·82	3·06	1·64	10
			2·86	1·82	10
1·61	3·50	1·83	4·06	1·74	20
			4·26	1·60	20
2·0	3·39	1·96	3·92	2·20	20
	4·36	2·05	3·06	1·94	20
3·84	3·39	1·96	3·83	1·95	20
	4·36	2·05	3·72	1·98	20
5·0	6·11	2·27	8·00	1·95	20
			9·25	2·08	20
8·05			3·50	1·75	15
16·10	3·25	2·33	3·60	2·04	15
40·25	3·71	2·24	3·19	1·90	20
80·50			3·11	1·32	20

might be found. Using a range of concentrations of triiodothyroacetic acid from 0·96 to 80·5 μM and incubation times of 10, 15, 20 and 40 min. we have been unable to demonstrate consistent increases either in O_2 consumption or iodide uptake (Table 6).

Thyrotrophic hormone was added to the suspending medium in four concentrations ranging from 0·025 to 4·25 units (U.S.P.) without any significant effect on O_2 consumption or on uptake of $^{131}I^-$ by the slices. In this experiment the tissue was incubated for 105 min. instead of the usual 40 min., as it seemed likely from *in vivo* experiments that a longer period would be necessary to exclude an effect by thyrotrophic hormone.

Water content of tissue

The failure to depress T/M for $^{131}I^-$ below 0·8 suggested that the iodide of the medium was in diffusion equilibrium with the tissue water. The water content of the tissues was therefore determined. After incubation for 50 min. slices of gland were removed from the flasks, and blotted and weighed. They were then dried in an oven at 100° for 12 hr., and, after cooling, were reweighed. In duplicate experiments the ratio, weight of tissue water/weight of wet tissue, was 0·77 and 0·76.

DISCUSSION

These results show that the salivary gland of the mouse provides a relatively simple iodide-trapping system which can be studied *in vitro*. It resembles the iodide trap of the thyroid so closely in many respects that it is reasonable to suppose that the mechanism may be similar, but it can be studied more easily in the salivary gland as the iodide trap of the thyroid is intimately linked with the subsequent processes involved in the synthesis of organic iodine. There was no evidence that this occurred in the salivary gland during the period of these experiments, and the uptake of iodide by the gland was not affected by drugs such as methylthiouracil which act by inhibiting the synthesis of hormone. The relative efficacy of the anions ClO_4^-,

SCN^-, $^{127}I^-$ and NO_3^- in depressing the uptake of $^{131}I^-$ by the tissue slices was closely analogous to their effect on the iodide trap of the thyroid *in vivo* (Wyngaarden, Wright & Ways, 1952; Wyngaarden, Stanbury & Rapp, 1953) and *in vitro* (Freinkel & Ingbar, 1955), and on the secretion of radioiodine in human saliva (Edwards *et al.* 1954). The uptake of $^{131}I^-$ by the salivary gland is so sensitive to ClO_4^- that the tissue-slice technique could probably be used for biological estimation of this anion, which is notoriously difficult to estimate chemically; it has become important to study the pharmacology of this anion as it is now being used in the treatment of thyrotoxicosis.

Scatchard & Black (1949), using a pure solution of albumin, found that the combining powers of ClO_4^-, SCN^-, I^- and NO_3^- for protein were in the order $ClO_4^- > SCN^- = I^- > NO_3^-$. Fig. 2 shows that the concentration of iodide by the salivary slices satisfies the Langmuir adsorption equation and hence it is possible that adsorption of I^- is a stage in the trapping mechanism. Moreover, the similarity between the curves for ClO_4^-, SCN^- and I^- in Fig. 3, and the summation of the effects of ClO_4^- and SCN^- (Table 1) suggest that these ions act in a similar way and at the same point in the iodide trap. Competitive adsorption would thus appear to be a possible explanation of their inhibitory effects.

Our observations on the concentration of endogenous SCN^- in the tissue, however, seem to be in serious disagreement with this theory. Both ClO_4^- and $^{127}I^-$ failed to discharge the endogenous SCN^- from tissue slices, and only a proportion of it could be eluted even after an hour's immersion in a large volume of saline. On the other hand, although the tissue was almost saturated with endogenous SCN^-, the uptake of $^{131}I^-$ was inhibited by adding SCN^- to the suspending medium in a concentration which was small compared with that already present in the tissue. Moreover, the uptake of $^{131}I^-$ was not enhanced in tissue slices from which 60% of the endogenous SCN^- had been previously eluted by prolonged immersion in a large volume of saline. The most likely explanation of these findings is that the endogenous SCN^- in the tissue slices is not freely exchangeable with the medium and plays no part in inhibiting I^- uptake; experiments using radioactive SCN^- would show whether the salivary glands of the mouse contain a non-exchangeable pool of SCN^-. This high concentration of endogenous SCN^- may be peculiar to the mouse since the concentration in human and guinea-pig salivary glands is about the same as, or only a little above, that in the plasma. Wood & Kingsland (1950) were unable to demonstrate concentration of SCN^- by the thyroid tissue of the rat. Hence our experimental results with SCN^- are not necessarily inconsistent with the hypothesis of competitive adsorption of a variety of anions in the salivary gland. The observation that concentration of I^- by the salivary gland *in vivo* is not depressed by the relatively high amount of SCN^- present in the plasma could possibly be explained by inactivation of this SCN^- by binding to one of the plasma constituents.

Whatever the precise mechanism for concentrating I^- may be, the action of dinitrophenol in depressing both oxygen consumption and uptake of I^- indicates that high-energy phosphate bonds may provide most of the energy.

SUMMARY

1. It has been found that slices of salivary glands of the mouse concentrate [^{131}I]iodide 5–10 times from the surrounding medium.

2. All the ^{131}I in the gland is present as iodide; no evidence of organic synthesis has been obtained.

3. Concentration of [^{131}I]iodide is inhibited by the addition of perchlorate, thiocyanate, iodide and nitrate to the medium, the order of activity being perchlorate > thiocyanate = iodide > nitrate, as *in vivo*.

4. The amount of endogenous thiocyanate in the gland and plasma is high compared with the amount which has to be added to the medium to inhibit the concentration of [^{131}I]iodide.

5. The addition of 2:4-dinitrophenol to the medium depresses both the respiration of the slices and their concentration of [^{131}I]iodide, indicating that energy is probably necessary for the process of concentration.

6. Evidence is adduced for a possible competitive-adsorption process to explain the mechanism of concentration.

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Oligosaccharides Formed from Sucrose by Fructose-transferring Enzymes of Higher Plants

By P. J. ALLEN* and J. S. D. BACON
Department of Biochemistry, University of Sheffield

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The synthesis of oligosaccharides by the transfer of hexose residues from one molecule of sucrose to another has been observed with invertase preparations from yeast and filamentous fungi (see summary by Bacon, 1954*a*; also Gross, Blanchard & Bell, 1954; Bell & Edelman, 1954). The hexose residue transferred is fructose, resulting in the formation of oligosaccharides enriched in fructose. Fructose may also be transferred to other acceptor molecules with primary-alcohol groups, including a variety of sugars and alcohols (Bealing, 1953; Bacon, 1954*b*). Free glucose serves as an acceptor with both invertase preparations, though the disaccharides formed are different with yeast and mould enzymes (Edelman, 1954). Purification and other treatments of an invertase preparation lead to parallel changes in the hydrolytic and fructose-transferring activity, indicating that the two types of action are due to a single enzyme. An analogous transferring action has been observed in extracts of *Helianthus tuberosus*, but the enzyme involved is probably different from invertase, since inulin and oligosaccharides of the inulide series are preferential substrates for this enzyme (Edelman & Bacon, 1951; Dedonder, 1952*b*).

The products formed by yeast invertase acting on sucrose have been found to differ quantitatively and qualitatively from those produced by invertase from filamentous fungi, but some of the products are common to both types of enzyme. Table 1 gives some information about two of the latter; in the account that follows their 'abbreviated' names will be used. 1^F-β-Fructosylsucrose was crystallized by Barker, Bourne & Carrington (1954) and

* Present address: Department of Botany, University of Wisconsin, Madison, Wisconsin, U.S.A.

Table 1. *Trisaccharides from the action of invertase preparations*

Structure	Source of invertase	$[\alpha]_D$	Authors	Trivial names	Abbreviated names
O-α-D-Glucopyranosyl-(1→2)-*O*-β-D-fructofuranosyl-(1→2) β-D-fructofuranoside	Taka diastase	+27°*	Bacon & Bell (1953)	Component α_1 (Bealing & Bacon, 1953); 1-Kestose (Gross *et al.* 1954)	1^F-β-Fructosyl-sucrose
	Penicillium altramentosum	+29·0	D. Hibbert (personal communication, 1954)	—	—
	Aspergillus niger	+29·2	Barker *et al.* (1954)	Trisaccharide I	—
	Yeast	+27*	Bacon (1954*b*)	Component II_1	—
	Helianthus tuberosus†	+23*	Dedonder (1952*a*)	Glucofructosane B	—
	H. tuberosus†	+28·6	J. S. D. Bacon (unpublished, 1954)	Spot 2 (Bacon & Edelman, 1951)	—
	Beta vulgaris	+28·6	Present work	—	—
O-β-D-Fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1→2) β-D-fructofuranoside	Yeast	+21*	Bacon (1954*b*)	Component II_2	6^G-β-Fructosyl-sucrose
	Yeast	+22*	Gross *et al.* (1954)	*neo*Kestose	—
	Beta vulgaris	+21*	Present work	—	—

* Measured on an amorphous preparation.
† In *H. tuberosus* the substance is a normal constituent of the carbohydrates of the tuber; the invertase of this plant has not been investigated.

D. Hibbert (personal communication), but 6^G-β-fructosylsucrose is still available only in the amorphous state.

In the present paper, data are presented concerning the fructose-transferring properties associated with invertase activity in extracts from higher plants. It will be shown that two trisaccharides are the main products of fructose transfer by an enzyme preparation from the leaves of sugar beet (*Beta vulgaris* L.) and that these trisaccharides have properties identifying them with the two trisaccharides mentioned above.

METHODS

Enzyme preparations. An active enzyme preparation from sugar-beet leaves was obtained by squeezing the juice from fresh tissues through bolting cloth in a hydraulic press at pressures up to 2000 lb./in.^{2}. The petiole and main veins were dissected out and the juice was expressed separately from these tissues and from the remaining leaf blade. The juice was stored in Pyrex flasks at $-10°$ and did not change in invertase or in fructose-transferring activity during 10 months' storage. Samples were used either without further purification or after removal by centrifuging of material which precipitated during freezing and thawing. For some experiments the enzyme was slightly enriched and purified by partial freezing, decanting and dialysing in the cold against distilled water.

Other enzyme preparations were obtained by grinding tissue with a mortar and pestle and expressing the juice with the pestle, or by crushing frozen tissues in a Hughes press (Hughes, 1951). Grinding with sucrose did not detectably increase the activity of extracts. Prior treatment of leaves with ether vapour resulted in a greatly increased yield of juice with considerably reduced invertase activity.

Incubation of samples. Incubations were carried out at room temp. in stoppered flasks. The final concentration of substrate was usually 20% (w/v). For incubations of more than a few hours, $CHCl_3$ was added to prevent microbial growth. The pH values of the juices used ranged from 5·0 to 6·5. When adjustment of the pH was made, acetate buffer was employed. Before chromatograms of the reaction products were developed, the enzyme was inactivated with $HgCl_2$ placed on the starting line of the paper (Bealing & Bacon, 1953).

Measurement of radioactivity. This was carried out essentially as described by Edelman (1954). All papers were sprayed with benzidine–trichloroacetic acid (Bacon & Edelman, 1951) shortly before counting, and values obtained from the two sides of the paper were averaged.

Autoradiograms. These were prepared by placing the paper chromatograms in close contact with envelope-packed Ilford Industrial G X-ray Film (Ilford Ltd., Ilford, London) for periods of 10–40 days. The films were then developed for 6 min. in a D 19 B X-ray film developer (Kodak Ltd.) and fixed with an acid hardener. Papers which had been sprayed gave autoradiograms indistinguishable from those from unsprayed papers.

Paper chromatography. The apparatus and procedures were those described by Bacon & Edelman (1951). *n*-Butanol–acetic acid–water (Partridge, 1948), and Whatman no. 1 paper were used throughout. For routine detection of carbohydrates, the dried papers were sprayed with benzidine–trichloroacetic acid. The reducing properties of sugars were compared with those of suitable controls, chromatographed under the same conditions, by the use of the alkaline $AgNO_3$ reagents of Trevelyan, Procter & Harrison (1950) (see Bealing & Bacon, 1953; Edelman & Bacon, 1951).

Column chromatography. The products formed from sucrose after incubation with sugar-beet-leaf juice were separated by gradient elution from charcoal–Celite columns with increasing concentrations of ethanol (Alm, Williams & Tiselius, 1952) and examined according to the procedures described by Bacon (1954*b*).

Analytical procedures. Ketose was determined colorimetrically as described by Bacon & Bell (1948). Total reducing sugar before and after hydrolysis was determined by the method of Miller & Van Slyke (1936) with fructose as a standard (Bacon, 1954*b*). The reducing-sugar composition and optical rotation of the oligosaccharides were measured as described by Bacon & Edelman (1951). Melting-point determinations were made in capillary tubes inserted into an air space surrounded by an electrically heated jacket.

RESULTS

Separation and purification of two trisaccharides

When an extract of sugar-beet-leaf blades was incubated at pH 6·0 with 20% sucrose, chromatograms of the incubation mixture showed spots appearing in the glucose and fructose positions within a few hours and increasing in intensity for several days. After 6 hr. incubation, a third spot appeared behind the sucrose position, with an R_S (quotient R_F sugar/R_F sucrose) of approximately 0·6, and increased in intensity during the following week. On the basis of its R_S, this spot appeared to represent a trisaccharide. Usually a very faint spot also appeared after a day or two between this new component and sucrose. This spot, with an R_S value of about 0·75, never became very conspicuous and tended to disappear before the trisaccharide.

Extracts from sugar-beet petioles showed similar products after longer incubation with sucrose, but extracts from the root were almost completely lacking in activity. Hydrolysis of sucrose and formation of trisaccharide appeared to be as fast at pH 5·0 as at 6·0, but were both slower at pH 4·0.

To obtain material for further study of these products, 95 ml. of leaf-blade juice, 40 g. of A.R. sucrose, and 45 ml. of 0·2 M sodium acetate–acetic acid buffer, pH 5·0, were mixed (total vol. 160 ml.) and incubated in the presence of $CHCl_3$ for 8 days, during which time approximately half the sucrose was broken down. On centrifuging the mixture, a clear supernatant was obtained, which was fractionated on charcoal–Celite columns. A preliminary gradient elution of a 20 ml. sample resolved the mixture into four fractions. Paper chromatograms showed these to consist, in order of their appearance, of (1) glucose and fructose,

(2) sucrose, (3) and (4) two distinct components representing the single spot in the trisaccharide position. Of these two components, the one eluted first was present in large amount and had a slightly lower R_S value; a typical chromatogram, carrying three spots of each, gave mean R_S values of 0·606 and 0·620 for the earlier and later components respectively. In addition to these five sugars, trace amounts of two other sugars appeared between sucrose and the major trisaccharide.

The incubation mixture (125 ml.) was chromatographed on a B.D.H. 'activated' charcoal–Celite column (300 g.:300 g.; about 90 cm. high, 5·5 cm. in diam.), the eluates being collected on solid $BaCO_3$. The two trisaccharides were sharply separated. Each sample was filtered, evaporated to dryness *in vacuo* at below 40°, dissolved in 5·0 ml. of water, rechromatographed on Sutcliffe Speakman Active Carbon no. 130–Celite (50 g.: 50 g.), and the purified material again evaporated to dryness. The residues were further dried over $CaCl_2$ at 0·1 mm. Hg for 2 days, and weighed. At this stage, yields of 611 and 133 mg. respectively were obtained from about 31 g. of sucrose present in the original 125 ml. of incubation mixture. Each sample was again dissolved in an accurately measured quantity (5·0 ml.) of glass-distilled water, and the resulting solutions were used for further study as such, or after further purification.

Attempts to crystallize these sugars were successful with the major but not with the minor component. About 0·5 g. of the former was dissolved in 2 ml. of dry methanol and left in a sealed desiccator containing ethanol. The next day a non-crystalline precipitate had separated from the mixture of methanol and ethanol. Upon seeding with crystals of 1^F-β-fructosylsucrose (kindly provided by Mr D. Hibbert) the material crystallized. It was later recrystallized by dissolving in methanol with a trace of water and crystallizing as before, yielding 310 mg. of fine white crystals.

The major trisaccharide. On the basis of its reaction with the alkaline $AgNO_3$ and phloroglucinol sprays, this compound was non-reducing and contained ketose. The recrystallized material did not react to the Raybin (1933) test, which is essentially, though not exclusively, a test for derivatives of sucrose in which the fructose moiety is unsubstituted (see Levi & Purves, 1949). Upon slow hydrolysis with acetic acid (1 vol. of acetic acid added to 5 vol. of 10·8 %, w/v, solution of trisaccharide) two products appeared early and increased in concentration together. These two corresponded in position on paper chromatograms with sucrose and fructose. A third product, appearing later in the hydrolysis, corresponded chromatographically with glucose. No other products were detected at any stage. These results are consistent with a structure for the trisaccharide in which an extra fructose residue is linked to the fructose moiety of sucrose. The glucose which arose during the later stages of the hydrolysis could have come either from the sucrose liberated earlier or from direct hydrolysis of the trisaccharide, in which case a fructosylfructose would have been formed; this substance, which has almost the same R_F value as sucrose, was not looked for.

Analysis of the original syrup, and of the recrystallized material, for ketose content indicated a ratio of 2 ketose:1 non-ketose residue, when calculated on the basis either of dry weight or of total reducing sugars after hydrolysis with 0·5 % oxalic acid at 100° for 25 min. (Table 2).

The $[\alpha]_D^{20}$ of the recrystallized sugar, based on dry weight, was 28·6° in water; based on ketose content it was 29·1°. Its melting point was sharp at 200° before and after recrystallization. Decomposition occurred rapidly at this temperature after the crystals had melted, but there was no evidence of decomposition before melting.

Dr D. Gross has examined this substance by electrophoresis in borate buffer (see Gross, 1954; 4000 v, 46 mA, 2 hr.); it has the same mobility as 1^F-β-fructosylsucrose.

The minor trisaccharide. Like the major trisaccharide, this component was a non-reducing ketose-containing sugar. It gave a positive Raybin test, however. Slow hydrolysis with acetic acid led to the immediate appearance and rapid accumulation of sugars chromatographically identical with fructose and sucrose and of a third component travelling between the sucrose and the parent trisaccharide positions in *n*-butanol–acetic acid–water. This substance was a reducing sugar, which could arise from a non-reducing trisaccharide containing a sucrose residue only by splitting of the 1:2-glycosidic linkage between glucose and fructose. Since its accumulation preceded the appearance of glucose, hydrolysis of the 1:2-linkage must not have released free glucose. It therefore seemed probable that this reducing disaccharide was a fructosylglucose containing a fructose residue transferred during the original incubation with the leaf juice. That the substance contained both sugars was confirmed as follows: three spots of the partially hydrolysed trisaccharide were chromatographed, after which the paper containing the outer spots was cut out and sprayed to locate the position of the reducing disaccharide on the unsprayed portion. This was then cut out and eluted on to the starting line of a second paper with a concentrated solution of yeast invertase, according to the method of Zimmermann (1953). The second paper was developed, and sprayed with alkaline $AgNO_3$. Hydrolysis of the disaccharide had occurred during elution, and comparable amounts

Table 2. *Analysis of trisaccharide fractions*

Preparation	Sample taken (mg.)	Total reducing substance (mg.)	Ketose (mg.)	Ketose:non-ketose, on basis of		Purity, on basis of			$[\alpha]_D^{20}$ on basis of	
				Dry wt.	Total reducing substance	Total reducing substance (%)	Ketose* (%)		Dry wt.	Ketose†
Major trisaccharide										
(i) Syrup, original preparation	183·3	182·6	115	2:1·42	2:1·18	93·0	87·8		25·7°	29·0° (12·22)
(ii) (i) filtered and re-dried	324·5	314·4	201·5	2:1·45	2:1·12	90·5	86·9		25·8	29·4 (10·82)
(iii) Recrystallized	102·1	116·0	71·6	2:1·06	2:1·24	106	98·3		28·6	29·1 (2·04)
Minor trisaccharide										
(i) Syrup, original preparation	177·4	177·8	107·8	2:1·53	2:1·30	93·5	85·1		20·9	24·6 (4·43)
(ii) (i) diluted 1·5 ×	—	—	—	—	—	—	—		22·0	25·8 (1·96)
(iii) (ii) filtered and re-dried	80·5	84·3	56·0	2:1·08	2:1·01	97·7	97·4		20·6	21·2 (1·77)

* Assuming composition 1 aldose:2 ketose.
† Figure in parentheses is concn., as % (w/v), of solution used for measuring rotation.

of two reducing sugars were produced, running in the fructose and glucose positions. This fragment of the trisaccharide is probably identical with the fructosylglucose (component I) formed during the action of yeast invertase on sucrose (Bell & Edelman, 1954).

Ketose determinations yielded values of approximately 2:1 for the ketose:non-ketose ratio in this trisaccharide also (Table 2). The $[\alpha]_D^{20}$ of the purest preparation obtained was 20·6° in water on a dry-weight basis, 21·2° on the basis of ketose content.

The infrared absorption spectrum of a preparation made by precipitating the sugar from methanolic solution with ether was examined by Drs S. A. Barker and E. J. Bourne, and compared with that of 6^G-β-fructosylsucrose. They report: 'The trisaccharide has a similar, but not identical, infrared spectrum to that of [6^G-β-fructosylsucrose]. The main difference is that [6^G-β-fructosylsucrose] shows an extra peak at 888 cm.$^{-1}$.'

Dr D. Gross has examined this trisaccharide electrophoretically under the same conditions as the major trisaccharide and finds that it has the same mobility as 6^G-β-fructosylsucrose.

Action of sugar-beet-leaf enzymes on various oligosaccharides

The action of the leaf extract at pH 6·0 was tested against 20% (w/v) solutions of maltose, melezitose, trehalose, and the major trisaccharide described above, and against 10% (w/v) solutions of cellobiose and raffinose and a 20% (w/v) suspension of soluble starch, and qualitative comparisons were made with the activity against sucrose. On paper chromatograms, no detectable products were formed from starch or melezitose during 6 days' incubation. From maltose and from cellobiose traces of glucose and of a trisaccharide were formed. Trehalose, raffinose, sucrose and the trisaccharide were acted on much more rapidly than the other oligosaccharides. The only product appearing from trehalose was glucose. From the trisaccharide, substances travelling in the fructose and sucrose positions were first evident, later glucose and a new substance with R_S 0·30, probably a tetrasaccharide. This trisaccharide can thus serve both as substrate and acceptor for the enzyme. Raffinose yielded four products, all of which were clearly evident after 1 day. Three of these were chromatographically identical with fructose, glucose and galactose respectively. The fourth had an R_S of 0·62 and contained no ketose residue. It was therefore probably melibiose, which would be left upon removal of fructose from the raffinose molecule. Some transferring action to raffinose was indicated by the appearance at later stages of a spot in the tetrasaccharide position. Only traces of sucrose appeared at any stage in the incubation.

Transfructosylation to free glucose

Since raffinose was split chiefly by removal of fructose, and very little sucrose accumulated, the possibility presented itself of determining directly whether glucose might serve as a fructose acceptor with consequent synthesis of sucrose. Incubations of a dialysed leaf-juice preparation containing 10% (w/v) of raffinose, with or without 10% (w/v) of glucose, showed no evidence of component I and only traces of sucrose, which was formed equally whether or not glucose was present. No significant transfer from raffinose to glucose was indicated.

The question was further investigated by experiments to determine whether radioactivity became incorporated into sucrose or component I when sucrose was incubated with the enzyme in the presence of [^{14}C]glucose (cf. Edelman, 1954). A difficulty was encountered in these experiments: the chromatograms of uniformly labelled [^{14}C]glucose (obtained from the Radiochemical Centre, Amersham) showed some radioactivity in the di- and tri-saccharide positions. This amounted to about 2% of the total counts from the paper; autoradiograms showed it to consist partly of an unspecific trail, and partly of discrete spots, one of which might correspond with isomaltose/gentiobiose (Bacon & Bacon, 1954). When the experiments of Edelman (1954) with Taka diastase were repeated the incorporation of radioactivity into sucrose was found to be of such a magnitude that this contamination could be disregarded, but preliminary experiments with the sugar-beet enzyme indicated that incorporation, if any, was much less, and was obscured by the 'blank'.

After discussions with Dr Edelman, who had encountered similar difficulties, the following procedure was adopted: ten spots, each of 2·5 μl. of [^{14}C]glucose in 2% glucose solution (a solution made by dissolving 1·3 mg. of [^{14}C]glucose, having 100 μc radioactivity, in 0·5 ml. of 2% (w/v) glucose), were disposed at intervals of 0·5 cm. along the starting line of a strip of paper 17·5 cm. wide.

A guide spot of inactive glucose was placed 3·5 cm. away from either end and the chromatogram developed with *n*-butanol–acetic acid–water overnight (15–20 hr.). The paper was dried in a current of air at 50°, and strips carrying the guide spots were cut out, leaving the radioactive material on a strip 10·5 cm. wide. After the guide strips had been sprayed with benzidine–trichloroacetic acid, the chromatogram was reassembled and two lines, about 1·5 cm. apart, were drawn across the sheet within the fringes of the guide spots, but including the greater part of the glucose. The strip so formed was cut out, a point cut at one end, and elution with water begun in the open air of the laboratory after the manner of a descending paper chromatogram. Evaporation from the strip was such that no free water accumulated at the tip. After 0·5–1·0 hr. under these conditions the apparatus was covered by an inverted jar; a drop of water began to accumulate at the point of the paper. In some cases it was necessary to place some damp filter paper in the jar to bring this drop to the size at which it would fall off. Measurement of radioactivity showed that a drop (25–30 mg.) produced in this way contained 90% or more of the glucose applied to the chromatogram; it was essential to allow evaporation during the elution, a drop collected by elution in a humid atmosphere containing only about 10% of the glucose.

In a typical experiment 25 mg. of sucrose was dissolved in 0·025 ml. of 0·2M sodium acetate–acetic acid buffer, pH 5, and a drop (29 mg.) of the purified [^{14}C]glucose eluate added, followed by 0·15 ml. of the sugar-beet enzyme preparation. Samples of 5 μl. were chromatographed on paper and the distribution of radioactivity was studied by direct counting of successive 0·5 cm. levels of the chromatogram, and by autoradiograms. Table 3 shows the changes in the radioactivity of the sucrose position during incubation at 20°. (From the appearance of chromatograms sprayed with benzidine–trichloroacetic acid it was estimated that between one-half and three-quarters of the sucrose had been broken down in 145 hr.) Autoradiograms (3 or 5 μl. spots exposed for 3–5 weeks) showed the presence of four radioactive spots in the di- and tri-saccharide regions. One of these coincided exactly with the sucrose spot as revealed by the benzidine–trichloroacetic acid spray. Another, with R_F value somewhat smaller than that of sucrose, coincided with a very faint spot revealed by the

Table 3. *Incorporation of radioactivity into sucrose during incubation of sugar-beet-leaf enzyme preparation with sucrose and* [^{14}C]*glucose*

Reaction mixture (details in text) incubated at room temp. in Agla micrometer syringe. Radioactivity expressed as counts/min. from paper carrying the equivalent of 5 μl. of incubation mixture.

Time (hr.) ...	0*	1·7	4·5	28	47	99	145
Sucrose	0	3	7	17	22	35	32
Glucose	6281	—	6328	—	—	—	—

* Actually 10 min. No significant radioactivity was counted anywhere on this chromatogram apart from the glucose spot; 5 μl. of the reaction mixture placed on paper, but not run, gave 6434 counts/min.

spray. This radioactive spot appeared with equal intensity in autoradiograms of incubation mixtures in which the sucrose was replaced by fructose; it did not appear when the enzyme preparation was incubated with radioactive glucose alone. It therefore seems possible that this corresponds to component I. The remaining two radioactive spots did not correspond with any spots revealed chemically; they lay above and below the trisaccharide spot, in which there appeared to be negligible radioactivity, as would be expected if it were produced from the very feebly labelled sucrose. They may have been formed by fructose transfer from sucrose to the hypothetical radioactive component I, which would be expected to be of high specific activity.

Radioactive sucrose was formed only when sucrose was the substrate; in incubations with radioactive glucose alone the glucose remained throughout the only spot revealed by autoradiograms.

Transfructosylation with other plant juices

Oligosaccharide formation from sucrose was catalysed by juices extracted from the leaves, shoots and roots of other plants, and was always related in intensity to the invertase activity of the preparation. (The preparation of highest activity produced visible trisaccharide in 3 hr.) The extracts examined and showing formation of upper spots included those from leaves of broad bean (*Vicia faba* L.), thistle (*Cirsium arvense* Scop.), cow parsnip (*Heracleum sphondylium* L.), elder (*Sambucus nigra* L.), coltsfoot (*Tussilago fafara* L.), white clover (*Trifolium repens* L.), cabbage (*Brassica oleracea* L.), and mangel-wurzel (*Beta vulgaris* L.), and from young shoots of thistle and root tips of broad bean; an active extract was also made from the only monocotyledon examined, wheat (*Triticum vulgare* Vill.). Extracts without invertase or oligosaccharide-forming activity were obtained from the roots of sugar beet and mangel-wurzel, the cotyledons of broad bean, from apple fruit and the leaves of ivy (*Hedera helix* L.). The last two extracts were more acid than the others (pH 2–3 instead of 5·0–6·5) and the ivy-leaf extract was very viscous.

In all preparations the only conspicuous spot formed had an R_g value comparable with that of the trisaccharides from sugar-beet-leaf preparations. Traces of higher oligosaccharides were formed in some wheat-leaf preparations, and from cow parsnip and broad bean there was a transient and slight production of another sugar, running between the trisaccharide and sucrose positions.

DISCUSSION

The evidence presented here shows that an invertase preparation from sugar-beet leaf has transfructosylase activity, and that in the presence of sucrose the main product of transfer is the trisaccharide 1^F-β-fructosylsucrose, already known as a product of the action of yeast and mould invertases, and a normal constituent of the Jerusalem-artichoke tuber. The other trisaccharide is probably 6^G-β-fructosylsucrose, although a final identification must await the crystallization of this substance or a derivative.

Qualitative experiments with other higher plants suggest that the sugar-beet enzyme is typical. The differences between the three types of fructosaccharases are summarized schematically in Fig. 1. Like yeast invertase, the invertase of higher plants shows considerably more hydrolytic than transferring action.

In view of the suggestion (cf. Kurssanov, 1941) that invertase is part of the system responsible for the synthesis of sucrose in leaves, we have investigated the possibility that sucrose might be formed by fructose transfer to free glucose. Edelman & Bealing (1953) showed that mould invertase catalyses a rapid incorporation of radioactive glucose into sucrose. Yeast invertase, on the other hand (Edelman, 1954), catalyses its incorporation into a different fructosylglucose, probably 6-β-fructosylglucose (Bell & Edelman, 1954).

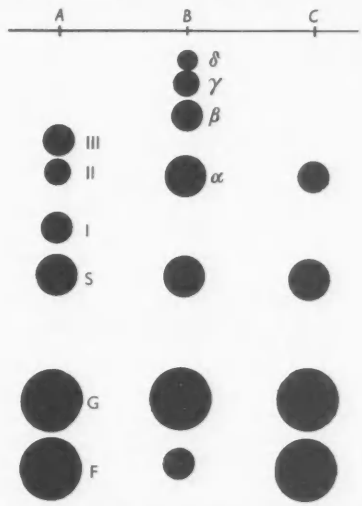

Fig. 1. Diagrammatic representation of intermediate stages in the breakdown of sucrose by invertase preparations from various sources (*A*, yeasts; *B*, moulds; *C*, higher plants), as revealed by paper chromatography in *n*-butanol–acetic acid–water. The sizes of the spots indicate approximately the amounts of the various compounds present. The lettering of the spots is according to Bacon & Edelman (1950) for yeast invertase, and Bealing & Bacon (1953) for mould invertase.

With the sugar-beet-leaf preparation the incorporation of radioactivity into the sucrose position of chromatograms was so slight that it was not practicable to confirm it by isolation of sucrose. However, the autoradiograms and the results of control experiments suggest that incorporation into sucrose is taking place, and that it is not due to a simple reversal of hydrolysis. By comparison with the mould enzyme, the activity is so small that it is difficult to believe that it can have any physiological significance. It is possible that yeast invertase may also have the ability to form sucrose to this extent, because the experiment described by Edelman (1954, Table 8) does not exclude incorporation of the magnitude found in our experiments.

Unlike the invertase of filamentous fungi, the preparations from higher plants did not form significant amounts of tetra- or higher oligosaccharides. Some other explanation must therefore be sought for the higher fructose-containing oligosaccharides found in such monocotyledons as wheat (White & Secor, 1953), barley (Porter & Edelman, 1952) and grasses (Laidlaw & Reid, 1952), or in the Compositae (Bacon & Edelman, 1951; Dedonder, 1952*a*). On the other hand, if due precautions are not taken to inactivate the invertase, trisaccharides may arise as artifacts in plant extracts.

SUMMARY

1. The action of aqueous extracts of higher plants on sucrose, and on other di- and tri-saccharides, was studied by paper chromatography. In all cases hydrolysis of sucrose was accompanied by the formation of trisaccharides.

2. The trisaccharides produced from sucrose by an enzyme preparation from the leaves of sugar beet were isolated and tentatively identified as *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2) β-D-fructofuranoside and *O*-β-D-fructofuranosyl-(2 → 6)-α-D-glucopyranosyl-(1 → 2) β-D-fructofuranoside.

3. During the action of the sugar-beet-leaf enzyme preparation on sucrose in the presence of uniformly labelled [^{14}C]glucose some incorporation of radioactivity into the di- and tri-saccharide fractions was detected.

4. The significance of these results is discussed in relation to the hypothesis that leaf invertase participates in sucrose synthesis.

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A Rapid Ultraviolet Spectrophotometric Method for the Detection, Estimation and Identification of Barbiturates in Biological Material

By P. M. G. BROUGHTON
Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford

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Ultraviolet spectrophotometric methods for the determination of 5:5′-disubstituted barbiturates in biological material are based on the fact that these exist in three forms in solution: (1) an un-ionized form in acid solution, with almost no selective light absorption in the range 230–270 mμ.; (2) first ionized form at pH 9·8–10·5 with an absorption maximum at 240 mμ. but no minimum in the range 230–270 mμ.; (3) a second ionized form at pH 13–14 with an absorption maximum at 252–255 mμ. and a minimum at 234–237 mμ.

Walker, Fisher & McHugh (1948), Lous (1950, 1954), Wright & Johns (1953) and others have used methods in which the light extinction was measured at 240 mμ. at pH 10 and in acid solution. Barbiturate was detected by the finding of an absorption maximum at this wavelength at pH 10, and its concentration was proportional to the difference, $E_{pH\,10} - E_{acid}$. Salicylic acid and sulphonamides also absorb in this region of the spectrum, and may therefore interfere with the detection and estimation of barbiturate by these methods. Walker *et al.* (1948) and Wright & Johns (1953) found that recovery of barbiturate, added to blood and determined by their methods, was low (55–70%), and applied correction for this. Born (1949) noted that blood extracts, prepared by the method of Walker *et al.* (1948), may become turbid after the addition of acid, and the present author has confirmed this.

Goldbaum (1948, 1952) developed a method for use with blood, urine and tissues, in which barbiturate was detected by the characteristic absorption spectra at 228–270 mμ. in solutions at pH 13 and at pH 10. The extinction difference, $E_{pH\,13} - E_{pH\,10}$, at 260 mμ., was proportional to barbiturate concentration, and in solutions containing no barbiturate $E_{pH\,13} = E_{pH\,10}$. This method appeared to be specific and gave good recoveries of added barbiturate, and has therefore been used as a basis for the work reported here.

Methods currently used for the identification of particular barbiturates extracted from biological material are usually slow and insensitive. Wolff (1951) developed a series of micro tests, some of which depended on qualitative differences in the extent of alkaline hydrolysis of different barbiturates under standard conditions. Goldbaum (1952) mentioned that attempts were being made to differentiate closely related barbiturates by their rates of decomposition in alkali, and described a method of distinguishing different barbiturates by comparing ratios of $E_{pH\,13} - E_{pH\,10}$ at different wavelengths.

This paper describes a critical examination of the variables in Goldbaum's (1952) method, and a modification is recommended for the detection and estimation of barbiturates in biological material. The influence of other materials on the absorption spectra obtained in this method has been investigated and the interpretation of these spectra is discussed. A method has been developed for the partial identification of barbiturates by measurement of the amount of alkaline hydrolysis under standard conditions.

EXPERIMENTAL

Materials. A.R. grade chemicals were used. The pure barbiturates used as standards are listed in Table 1, and were obtained commercially. These may be prescribed as either the acid or the sodium salt, and all concentrations reported in this paper are of the free dibasic acids.

Method

Barbiturate present in blood was extracted with $CHCl_3$, which was then extracted with 0·45N-NaOH. The absorption spectrum of the barbiturate in the clear alkaline solution was measured at pH 13·4 and at pH 10 (approx.) from 228–265 mμ. in 1 cm. silica cells, with a Unicam SP. 500 spectrophotometer.

Reagents. 0·6M-H_3BO_3–KCl; 0·5M-K Na phosphate buffer, pH 7·4; 0·45N-NaOH; $CHCl_3$; borate blank solution, prepared by mixing equal vols. of 0·45N-NaOH and 0·6M-H_3BO_3–KCl.

Extraction. Whole blood (5–10 ml.) is extracted three times with about 30 ml. of $CHCl_3$; the use of insufficient $CHCl_3$ tends to produce emulsions. The combined $CHCl_3$ extracts are filtered through an 11 cm. Whatman no. 31 filter paper into a separating funnel, and the paper is washed with a little $CHCl_3$. The $CHCl_3$ solution is extracted with 0·45N-NaOH (usually 5 ml., but 10 ml. if the rate of alkaline hydrolysis is to be measured) by vigorous shaking for 1 min. After separation of the phases the aqueous layer is clarified by centrifuging.

With urine, gastric contents and other liquid samples it is often advisable to take larger volumes (i.e. 10–25 ml.). The sample is acidified with H_2SO_4 and extracted as described above and the combined $CHCl_3$ extracts are washed twice with 5 ml. of phosphate buffer before filtering. Blood extracts suspected of containing salicylic acid or sulphonamides should also be washed twice with phosphate.

Tissues may be extracted by homogenizing a weighed sample (1–5 g.) with $CHCl_3$ and then proceeding as described for urine.

Hydrolysis. The clear 0·45N-NaOH extract (5 ml., preferably containing more than 100 μg. of barbiturate) is heated in a tube (calibrated at 5 ml.) in a boiling-water bath for exactly 15 min., and cooled rapidly in cold water and made up to 5 ml. with distilled water. The solution may froth slightly at the beginning of the heating owing to volatilization of traces of $CHCl_3$.

Measurement. Portions (2 ml.) of the 0·45N-NaOH extract are added to 2 ml. of 0·45N-NaOH and to 2 ml. of 0·6M-H_3BO_3–KCl. The extinctions, E_N and E_B, of these two solutions are measured from 227 to 265 mμ., with 0·45N-NaOH as a blank for the former and the borate-blank solution for the latter. Particular attention should be paid to the wavelengths at which maxima, minima and isosbestic points occur (Fig. 1). It is advisable to measure the extinction of the borate solution first; if necessary this solution must be diluted with the borate-blank solution until the extinction at 240 mμ. is, preferably, 1·0–1·5. Measurement of the extinction at other wavelengths and reference to Fig. 1 will often enable this dilution to be found. The NaOH solution must be diluted with 0·45N-NaOH by the same amount before measurement.

The same procedure is used with the solution after hydrolysis.

Calculation and interpretation of results. The criteria for the spectrophotometric detection of barbiturates are as follows (see Fig. 1): maximum at 238–240 mμ. in borate; maximum at 252–255 mμ. and a minimum at 234–237 mμ. in NaOH; isosbestic points at 227–230 mμ. and at 247–250 mμ. The greatest differences between E_N and E_B are at 260 mμ. (positive) and 236 mμ. (negative).

If all these criteria are obtained barbiturate may be said to be present. Dilute solutions (Fig. 4), however, may not give all these criteria and other compounds (see Figs. 5 and 6) may give some of them. Proof of the presence of barbiturates in such solutions must therefore depend on the absorption spectrum obtained; the mere finding of an extinction difference at 260 mμ. is not such a proof.

If c is the concentration of barbiturate (μg./ml.) in the solution in the spectrophotometer cell, and K_N and K_B are the specific extinction coefficients of the barbiturate in NaOH and borate solution respectively, then $E_N = K_N c + x_N$ and $E_B = K_B c + x_B$, where x_N and x_B represent absorptions due to materials other than barbiturate. At 260 mμ. $x_N \simeq x_B$ (Fig. 2), so that at this wavelength $E_N - E_B = c(K_N - K_B)$ or $c = F(E_N - E_B)$, where $F = 1/(K_N - K_B)$, and is independent of concentration (Table 1).

The barbiturate may be partially identified by measuring the percentage of barbiturate remaining, R, after alkaline hydrolysis, and using values of R given in Table 1. A complete analysis, including alkaline hydrolysis and calculation of results, may be made within an hour.

RESULTS

Detection and estimation of barbiturate

Fig. 1 shows the absorption spectra at two pH values of a barbitone solution. The solution in 0·45N-NaOH (pH 13·4, Ingles & Israel, 1948) has a maximum at 255 mμ. and a minimum at 234 mμ., and the borate solution (pH approximately 10) has a maximum at 239 mμ. The two curves cross at 227 mμ. and at 249 mμ. Absorption spectra at intermediate pH values (not reproduced) also pass through these points, which are therefore isosbestic points. The maximum positive difference between the extinctions of the NaOH and borate solutions of barbiturate is at 260 mμ. All other 5:5′-disubstituted barbiturates tested gave spectra with these maxima, minima and isosbestic points.

Absorption spectra at these two pH values of a blood extract containing no barbiturate is given in Fig. 2. Extracts from other biological materials gave similar spectra, and in each case $E_N \simeq E_B$ at 260 mμ. This wavelength appeared to be suitable for the estimation of barbiturates.

Effect of NaOH *concentration on* E_B. At 260 mμ. phenobarbitone gave constant values of E_N in

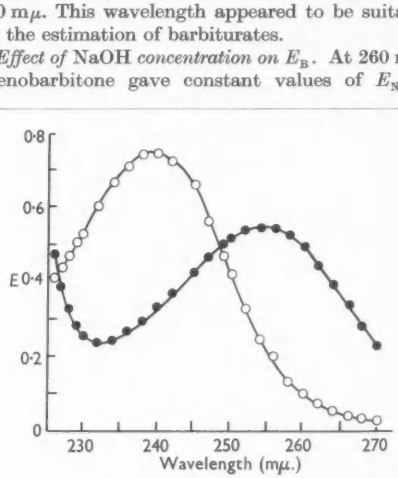

Fig. 1. Absorption spectra of a barbitone solution (13·5 μg./ml.). ●, In 0·45N-NaOH; ○, in borate.

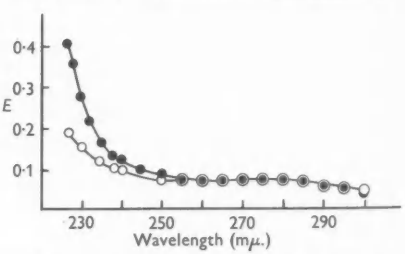

Fig. 2. Absorption spectra of a normal blood extract. ●, In 0·45N-NaOH; ○, in borate.

NaOH solutions stronger than 0·42N, but E_N of quinalbarbitone solutions had not reached a maximum in 0·54N-NaOH. Other barbiturates tested were intermediate between these two. It was considered that 0·45N-NaOH was a suitable solvent for the measurement of E_N, although it was recognized that the dissociation of quinalbarbitone from first to second ionized forms, indicated by the increase of E_N at 260 mμ., was not complete in this solution.

*Effect of boric acid–*KCl *concentration on* E_B. Boric acid–KCl solutions stronger than 0·8M tended to crystallize at room temperature. When solutions weaker than 0·5M were mixed with equal volumes of barbiturate in 0·45N-NaOH the extinctions, E_B, of the mixtures at 260 mμ. were higher than those obtained with the more concentrated boric acid–KCl solutions, indicating that the barbiturate had not been completely converted into the first ionized form because of insufficient decrease in pH. Suitable readings of E_B were given with 0·6M boric acid–KCl, and the pH of the mixture measured with the glass electrode was 9·9.

Effect of NaOH *concentration on* E_B. When equal volumes of 0·6M boric acid–KCl were mixed with barbiturate solutions of varying NaOH concentration the extinction E_B was constant for 0·34–0·56N-NaOH, but increased with higher concentrations of NaOH. Stable, buffered solutions, giving reproducible extinctions, could be obtained with 0·45N-NaOH and 0·6M boric acid–KCl, and these were therefore used in subsequent experiments.

Effect of wavelength on $E_N - E_B$. Results given in Fig. 3 indicate that $E_N - E_B$ reaches a flat maximum at about 260 mμ., and that minor errors in wavelength setting or calibration will not materially affect the measurement of the required value.

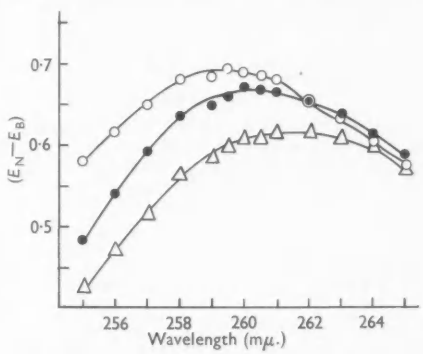

Fig. 3. Effect of wavelength on the extinction difference ($E_N - E_B$) of barbiturates (30 μg./ml.). ●, Phenobarbitone; ○, amylobarbitone; △, quinalbarbitone.

Calibration. E_N at 260 mμ. was measured for solutions of pure barbiturates in 0·45N-NaOH. These were then mixed with equal volumes of 0·6M boric acid–KCl and E_B was measured. A linear relationship between $E_N - E_B$ and barbiturate concentration was obtained, and values of K_N, K_B and F were calculated for the different barbiturates (Table 1). It should be noted that standard solutions of barbiturate in alkali are not stable.

Biological extracts containing no barbiturate

The determination of barbiturate depends on the fact that, at 260 mμ., $E_N = E_B$ for extracts containing no barbiturate (Fig. 2). In practice, however, small differences were obtained, and analysis of twenty-seven normal urines gave a mean value of $E_N - E_B = +0{\cdot}003$, S.D. $\pm 0{\cdot}031$. This would be equivalent to an apparent barbiturate concentration in the original urine of $\pm 0{\cdot}25$ mg./100 ml. (± 2 S.D.). Normal blood gave similar results.

Biological extracts containing barbiturates

Absorption spectra of extracts from urines containing varying amounts of added barbitone are given in Fig. 4. At small concentrations (0·48 mg./100 ml.) no maxima were obtained (Fig. 4*a*). This spectrum could not be said to prove the presence of barbiturate, but the crossing of the two curves is suggestive when compared with that of a normal extract (Fig. 2). At higher concentrations (0·96 mg./100 ml.) there was a maximum or shoulder at 238–240 mμ. in borate (Fig. 4*b*). Urine containing 6·5 mg. of barbitone/100 ml. gave the typical barbiturate spectrum (Fig. 4*c*): i.e. two isosbestic points, in borate a maximum at 240 mμ., and in NaOH a minimum at 235 mμ. and a maximum at 252 mμ. The precise concentration at which this typical spectrum is obtained cannot be stated, since the amount of general background absorption varies with different extracts and is usually greater for urine than for blood. Increasing the volume of sample extracted will increase the level of this absorption. Characteristic spectra have, however, been obtained with extracts from the following materials: blood containing 0·8 mg. of barbiturate/100 ml.; stomach washings, 0·2 mg./100 ml.; urine and stomach contents, 1·8 mg./100 ml. Spectra similar to Fig. 4*c* have been obtained with extracts of blood, urine, tissues and other pathological material from patients with barbiturate poisoning.

When the seven barbiturates listed in Table 1 were added to blood and urine and determined by the method given, recoveries were good for all except barbitone, for which recoveries were low (mean 90%). It appeared that this material was not completely extracted from $CHCl_3$ by 0·45N-NaOH. Accordingly, the method has been calibrated for barbitone by extracting known amounts, whereas

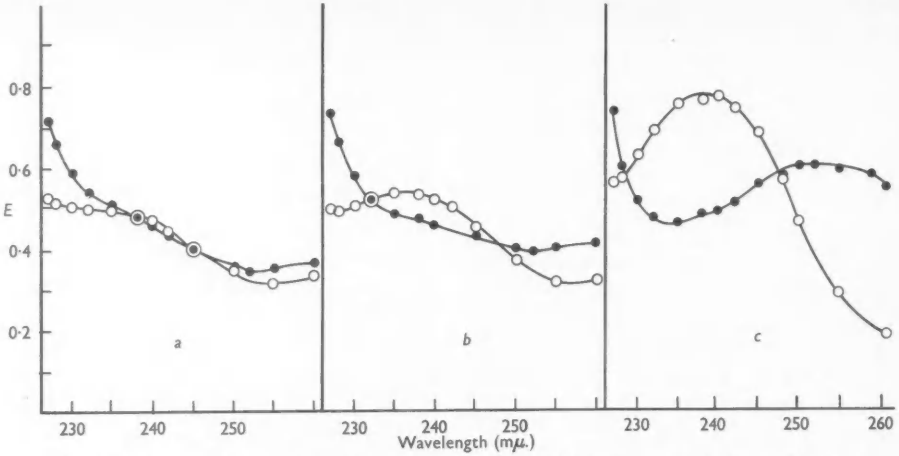

Fig. 4. Absorption spectra of extracts of urines containing added barbitone. *a*, 0·48 mg./100 ml.; *b*, 0·96 mg./100 ml.; *c*, 6·5 mg./100 ml. ●, In 0·45 N-NaOH; ○, in borate.

other barbiturates were calibrated directly. Results were then satisfactory, as shown by the following example: barbitone was added to blood to give a concentration of 5·0 mg./100 ml. Eleven separate determinations were made, and 10 ml. of blood was extracted by the recommended method. The mean concentration found was 4·82 mg./100 ml., S.D. ± 0·21 mg./100 ml. The mean ± twice the standard deviation is therefore equivalent to a recovery of 88–105 %. The accuracy of the method will obviously depend on the actual value of the extinctions measured.

There was no significant difference between recoveries of barbiturate added to blood and urine, and washing of the extract with phosphate buffer did not result in significant loss of barbiturate.

Interfering materials

The absorption spectra of salicylic acid and sulphanilamide in 0·45 N-NaOH, borate and acid solutions are given in Figs. 5*a* and 6*a*. At 260 mμ. E_N is not equal to E_B, and these substances may therefore interfere in the determination of barbiturate unless previously removed.

Extracts of blood containing added salicylate and barbitone gave the absorption spectra given in Fig. 5*b*, with an apparent recovery of 140 % of the barbiturate. When the $CHCl_3$ extract of this blood was washed with phosphate buffer the typical barbiturate spectrum was obtained with a satisfactory recovery (85 %) of barbiturate. This treatment is therefore effective in removing salicylate. Blood and urine from a patient taking large quantities of salicylate by mouth (blood-plasma salicylate 35 mg./100 ml.) gave values of $E_N - E_B$

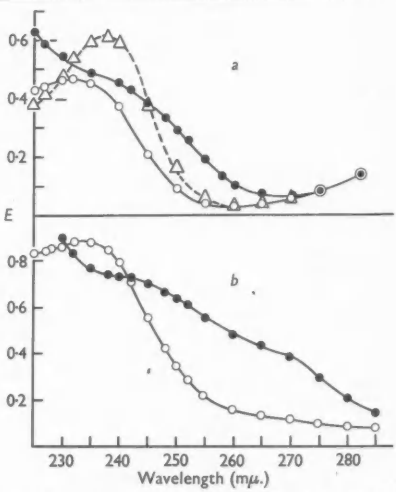

Fig. 5. Absorption spectra of *a*, 0·01 mg. of salicylic acid/ml., and *b*, an extract of blood containing 50 mg. of salicylic acid and 3 mg. of barbitone/100 ml. ●, In 0·45 N-NaOH; ○, in borate; △ (broken line), in 0·1 N-H_2SO_4.

within the normal range when extracts were washed with phosphate buffer, and it is concluded that salicylate and its metabolites do not interfere in the determination of barbiturate by this method.

Fig. 6*b* shows the absorption spectra of an extract of blood containing added sulphanilamide and butobarbitone. The sulphanilamide spectrum distorts that of the barbiturate and results in

apparently low recovery of the latter. When sulphanilamide was added *in vitro* this interference could be reduced by washing with phosphate buffer, but absorption spectra of extracts of urines from patients treated with sulphonamides still showed this interference. It was concluded that sulphonamides and their metabolic products will probably interfere with the determination of barbiturate, but that this may usually be recognized by the distortion of the barbiturate spectrum in the 240–260 mμ. range owing to absorption by sulphonamides at 250–260 mμ. in NaOH and borate.

Megimide (β-ethyl-β-methylglutarimide) has recently been recommended (Shulman, Shaw, Cass & Whyte, 1955) in the treatment of cases of acute barbiturate poisoning, and since the structure of this compound is similar to that of barbiturates, it was thought possible that it might interfere with the analysis of barbiturates. Megimide in 0·45N-NaOH has an absorption maximum at 230 mμ., and E_N is not equal to E_B at 260 mμ. In solutions containing less than 4 mg./100 ml. this extinction difference is not large enough to be measured as barbiturate, and the absorption maximum at 230 mμ. in NaOH is apparent at this concentration, even in the presence of barbiturate. Megimide decomposes rapidly in 0·45N-NaOH and its presence in extracts may therefore be confirmed by repeating extinction measurements on the NaOH solution 30 min. later. Megimide will react with cobaltous salts in a manner similar to that of barbiturates (Koppanyi, Dille, Murphy & Krop, 1934), and positive results have been obtained with this test when applied to extracts of urines containing more than 10 mg. of Megimide/100 ml.

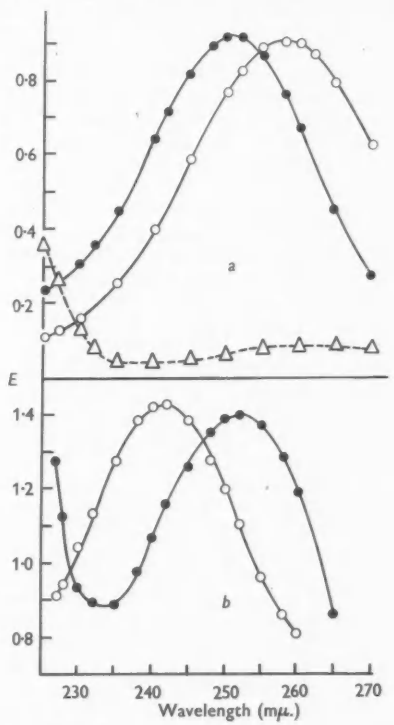

Fig. 6. Absorption spectra of *a*, 0·01 mg. of sulphanilamide/ml., and *b*, an extract of blood containing 10 mg. of sulphanilamide and 4 mg. of butobarbitone/100 ml. ●, In 0·45N-NaOH; ○, in borate; △ (broken line), in 0·1N-H_2SO_4.

Identification of particular barbiturates

Absorption spectra of the seven barbiturates tested were very similar, and no function of E_N and E_B could be found that would enable barbiturates to be identified. The decomposition of barbiturates in alkali was, however, successfully used in their partial identification. Solutions in 0·45N-NaOH were heated in a boiling-water bath and the percentage, R, of barbiturate remaining after a given time found by measuring $E_N - E_B$ at 260 mμ. before and after hydrolysis. After 15 min. hydrolysis R varied from 31·8% for phenobarbitone to 98·2% for pentobarbitone. With the more rapidly hydrolysed barbiturates the time of heating was critical, an extra minute producing up to 3% more destruction. The NaOH concentration at 0·45N was not critical, and R was independent of the concentration of barbiturate in the original solution. The validity of this method depends upon the fact that the products of the hydrolysis have $E_N = E_B$ at 260 mμ. After hydrolysis of a phenobarbitone solution containing 46·6 μg./ml. no barbiturate could be detected spectrophotometrically, and $E_N \simeq E_B$ at 260 mμ.

Values of R obtained after hydrolysis of pure barbiturate solutions for 15 min. are given in Table 1. This method has been applied to materials from patients with barbiturate poisoning and to normal bloods and urines containing added barbiturate; no significant differences were found between the results in these two types of sample. More than seventy specimens have been analysed by this method and the differences between the value of R found with biological extracts and the 'true' value (Table 1), obtained with pure solutions, had a standard deviation of $\pm 5\%$. Thus this method will not identify all the barbiturates listed, but it should at least distinguish the short, intermediate and long-acting ones. For example, in eleven separate analyses on blood containing added barbitone (5·0 mg./100 ml.) the mean value of R found was 46·5, S.D. $\pm 2{\cdot}3$. This result would not

Table 1. *Calibration data for 5:5′-disubstituted barbiturates*

K_N and K_B are specific extinction coefficients of barbiturate in NaOH and borate solutions at 260 mμ. F, calibration constant $[1/(K_N - K_B)]$. R, percentage barbiturate remaining after alkaline hydrolysis for 15 min. Recoveries of barbitone after $CHCl_3$ extraction were low (90%), and the values of K_N, K_B and F given for this substance were determined by extracting known amounts of barbitone.

Barbiturate	Substituent groups	Duration of pharmacological action*	K_N	K_B	F	R (%)
Phenobarbitone	Phenyl Ethyl	Long	0·0315	0·0086	43·7	31·8
Barbitone	Ethyl Ethyl	Long	0·0329	0·0062	37·5	42·5
Butobarbitone	*n*-Butyl Ethyl	Intermediate	0·0314	0·0056	38·8	49·6
Amylobarbitone	*iso*Pentyl Ethyl	Intermediate	0·0292	0·0052	41·7	55·8
Cyclobarbitone	Ethyl *cyclo*Hexenyl	Short	0·0302	0·0078	44·7	91·7
Quinalbarbitone	Allyl 1-Methylbutyl	Short	0·0288	0·0078	47·5	97·6
Pentobarbitone	Ethyl 1-Methylbutyl	Short	0·0268	0·0060	48·1	98·2

* Cf. *The Extra Pharmacopoeia* (Martindale, W.), 23rd ed., vol. I, p. 247 (London: The Pharmaceutical Press).

distinguish barbitone from butobarbitone, but does enable a partial identification to be made rapidly and on small quantities of material.

DISCUSSION

Detection

The reaction of barbiturates with cobaltous salts (Koppanyi *et al.* 1934) is widely used as a qualitative test. At least 0·2 mg. of barbiturate is necessary for a positive result and the method, which is by no means specific (Riley, Krause, Steadman, Hunter & Hodge, 1940), cannot be readily applied to blood.

Ultraviolet spectrophotometric methods are rapid and sensitive, and can be used with a wide variety of biological material. An absorption maximum at 240 mμ. at pH 10 is not, however, specific for barbiturates. Goldbaum (1952) showed that barbiturate could be detected by its characteristic absorption spectra at pH 13 and 10, and the present work has confirmed that this is specific. The method, and examples of the characteristic barbiturate absorption spectra, given in this paper may be used to prove the presence of barbiturate in biological material.

Estimation

Goldbaum (1952) did not give the complete experimental evidence for the choice of conditions used in his method. A systematic investigation of the variables in this method largely confirmed the choice of conditions made by Goldbaum. The use of 0·6 M boric acid–KCl, instead of 0·5 M, is recommended, since this increases the buffering of the solution and results in greater reproducibility of extinction readings E_B. Modifications in technique are recommended, and the proposed method is rapid, sensitive and specific.

Salicylic acid and sulphonamides do not have equal extinctions at 240 mμ. in solutions at pH 10 and in acid, and they may therefore interfere in methods for the determination of barbiturate, such as that of Walker *et al.* (1948), based upon extinction differences, $E_{pH\,10} - E_{acid}$, at 240 mμ. These authors recognized this fact, but it remains difficult to detect this interference from the absorption spectra obtained. Salicylic acid and sulphonamides should also interfere in the method of Goldbaum (1952) since $E_{pH\,13}$ is not equal to $E_{pH\,10}$ at 260 mμ. This author stated that sulphadiazine will not interfere, since $E_{pH\,13} = E_{pH\,10}$ for this compound at 260 mμ., but the work reported in the present paper shows that sulphonamides and their metabolites can interfere with this determination and their detection from the absorption spectra is described. Interference by Megimide in this determination may be recognized from the absorption spectra obtained and by its rapid decomposition in 0·45 N-NaOH.

Identification

With the development of quantitative methods it became obvious that the significance of a given blood barbiturate concentration depended on the type of drug present, and this determination was of limited value in poisoning cases unless the barbiturate could, at least, be partially identified

(Wright, 1954, 1955; Broughton, Higgins & O'Brien, 1956).

Of the methods generally used for the identification of barbiturates, organic analysis (Raventos, 1954) needs comparatively large amounts of material, colour reactions (Turfitt, 1948) are only reliable on carefully purified extracts, and paper chromatography (Algeri & Walker, 1952; Allgen, 1953; Wickström & Salvesen, 1952) uses 25–100 μg. of material, but takes 4–7 hr. for a good separation of barbiturates to be achieved. Wright (1954) used ultraviolet spectrophotometry for the determination, and paper chromatography for the identification, of barbiturates in biological material. Goldbaum (1952) found that barbiturates could be partially identified by measuring ratios of $E_N - E_B$ at various wavelengths, but the present author has been unable to confirm this.

Wolff (1951) used micro-analytical methods for the identification of the substituents of barbiturates, including alkaline hydrolysis to the dialkylurea derivatives, some of which had a characteristic crystalline appearance. The present method of identification is a quantitative development of this author's observations on the alkaline hydrolysis of barbiturates. It is intended as a rapid partial identification; further analysis by any of the methods described above may be made on the extracts after spectrophotometric measurements.

Metabolites of barbiturates, in which the pyrimidine ring is intact, have absorption spectra similar to those of the original compounds (Maynert & Dawson, 1952; Lous, 1954), but it is probable that they will be hydrolysed in alkali at a rate different from that of the original barbiturate. Values of R, determined on material obtained from patients with acute poisoning, do not differ significantly from values obtained with pure solutions, and this suggests that the influence of these metabolites is not sufficient to interfere in this method.

The hydrolysis constant R and, with one exception, the calibration constant F appear to be inversely proportional to the duration of action of the different barbiturates (Table 1). These facts are often useful in the analysis of specimens from cases of acute poisoning, where it may be sufficient to classify the barbiturate in this way, rather than to attempt complete identification. Results obtained with this method in cases of acute poisoning are discussed by Broughton *et al.* (1956).

SUMMARY

1. A rapid method is described for the determination of barbiturates in biological material by ultraviolet spectrophotometry.

2. Characteristic absorption spectra of barbiturates in varying concentration are given, and the criteria for their detection are listed. Absorption spectra of salicylate and sulphonamides, which can interfere with the determination, are given and their recognition or removal is described. Interference by Megimide (β-ethyl-β-methylglutarimide) in the detection of barbiturate by this method, and by its reaction with cobaltous salts, is described.

3. A method is described for the partial identification of barbiturates by measurement of the amount remaining after alkaline hydrolysis under standard conditions.

I wish to thank Mr J. R. P. O'Brien for advice and criticism during this work. I also wish to thank British Drug Houses Ltd. for a gift of cyclobarbitone and Nicholas Products Laboratories Ltd. for a gift of Megimide.

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The Choice of the Suspension Medium for Rat-Liver-Cell Nuclei

By J. ST L. PHILPOT and JEAN E. STANIER
Medical Research Council Radiobiological Research Unit, Atomic Energy Research Establishment, Harwell, Didcot, Berks

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The isolation and study of cell particles such as nuclei, mitochondria, etc., require a suspension medium which should be chosen so as to preserve intact the whole structure and function if possible, or at least the particular features being investigated. The natural medium, cell sap, is difficult to obtain free from extracellular fluid, and its composition is therefore largely unknown. Many artificial media have been advocated for nuclei (cf. reviews by Allfrey, Mirsky & Stern, 1955, and Dounce, 1954, 1955), and a surprising variety of abnormal appearances can be produced by conditions which are becoming increasingly well defined and which follow different rules for each abnormality. The present work is an attempt to define these conditions still better, and to design an aqueous artificial medium that satisfies several visual criteria of normality. These simple criteria alone suffice, without the aid of enzymic criteria, to exclude many of the media previously advocated, if complete normality is the aim.

The criteria of normality used in the present work are all based on observation with the phase-contrast microscope, and are as follows:

(1) The nuclei should be non-granular, i.e. their optical density should be uniform except for the nucleoli. This requires that the ionic strength of the medium should be below a critical value depending on the pH, the concentration of bivalent cations (e.g. Ca^{2+} and Mg^{2+}), the dielectric constant of the solvent, etc. (see Expt. 1). Moderate granularity seems to be reversible, and is therefore probably harmless. It may even be related to the condensation of chromosomes at mitosis. Nevertheless, for simplicity it is better avoided in experiments on resting nuclei.

(2) The nucleoli should be clearly visible and the nucleus should not be swollen (see Expt. 2). This sets a lower limit to the ionic strength, which is fortunately below the upper limit defined by criterion (1).

(3) The nuclei should not be clumped, even after being centrifuged and resuspended. The best way of satisfying this criterion seems to be by lowering the dielectric constant of the solvent and by keeping the ionic strength of the medium or the concentration of bivalent ions as high as is compatible with criteria (4) and (1) (see Expt. 3).

(4) The mitochondria should be sufficiently unclumped to be separable from nuclei by centrifuging. Mitochondrial, unlike nuclear, clumping increases with ionic strength and with concentration of bivalent ions, and there seems to be no known aqueous medium in which both kinds of clumping are completely prevented. The choice of ionic strength during the centrifugal separation is therefore determined by a careful balance between criteria (3) and (4).

(5) The nuclear membrane should be free from 'blebs': i.e. clear round bubbles attached locally to the membrane which apparently contain the medium in which they have formed, and which behave osmotically when transferred to another medium. Anderson (1953*a*), who first described blebs, found that they could be prevented by sucrose. We have found that glycerophosphate will also prevent them (see Expt. 4).

(6) When the nuclei are transferred to molar sodium chloride the deoxyribonucleic acid (DNA) should swell through the nuclear membrane, forming a halo, and thereby demonstrate that it is still present in soluble viscous form (see Expt. 5). The haloes are most easily seen in the presence of mitochondria or other small particles.

(7) The nuclear contents as judged by optical density should not diminish on standing. This can be prevented by sucrose, or better by glycerophosphate (see Expt. 6).

METHODS

Perfusion of liver. The livers of Wistar-strain rats anaesthetized with ether were perfused either via the portal vein through a glass cannula or via the left ventricle with a 30 ml. syringe, first with ice-cold 0·15 M-NaCl until all the blood was washed out, and then with ice-cold 0·29 M sucrose. 0·15 M-NaCl was found to wash out blood more completely than sucrose (cf. Dounce, 1955), possibly by keeping plasma globulins in solution. It was subsequently washed out with sucrose so that it did not contaminate the homogenizing fluid. The whole operation took about 10 min. Perfusion through the left ventricle was quicker but somewhat less effective than perfusion through the portal vein. The liver was removed and kept on ice in 0·29 M sucrose until it was needed. Storage in this way for 2 days did not alter perceptibly the appearance of homogenates, but except for preliminary experiments the perfused liver was always used within 4 hr.

Homogenization of liver. The liver was cut up with scissors and homogenized in a $\frac{1}{2}$ in. precision-bore glass tube closed by a rubber bung at the bottom, with a non-rotating stainless-steel conical plunger with a half-angle of 6°, widest at the bottom, where the diameter was 0·004 in. less than the bore of the tube (i.e. the gap was 0·002 in.) (Fig. 1). About seven strokes of the plunger gave maximum breakage of cells (71–76%). Homogenization was sometimes continued with a tube and plunger which gave a finer gap (0·001 in.). In this case, 76–85% of the cells were finally broken. The medium used for homogenization depended on the purpose of the experiment. Medium *A*, which gave normal preparations as judged by the criteria outlined above, contained: 0·3 M sucrose, 0·065 M potassium glycerophosphate, 0·001 M-$MgCl_2$ and 40% glycerol, pH 7·0. The liver tissue was diluted 1 in 5 or 1 in 10 (w/v) for homogenization. Since cells were less easily broken in glycerol-containing solutions, the glycerol was sometimes added after homogenization, by adding to the homogenate an equal volume of the homogenizing medium containing 80% glycerol. All the solutions used were kept at 0–5°.

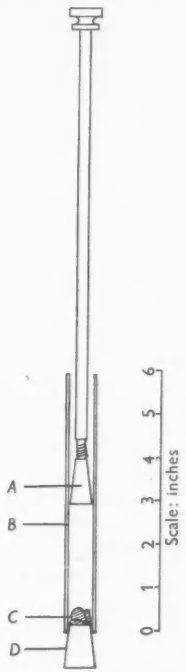

Fig. 1. Section of homogenizer: *A*, stainless-steel conical plunger; *B*, precision-bore glass tube; *C*, liver; *D*, rubber bung.

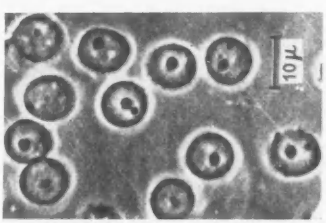

Fig. 2. Nuclear preparation in medium *A*. This and subsequent photographs were taken by using a Beck phase-contrast microscope with a 2 mm. objective.

Centrifuging. For the preparation of nuclei used in most of the small-scale experiments described in this paper, 2 ml. of the homogenate was layered over 8 ml. of medium *A*. This was possible since the density of rat liver is 1·086 (Anderson, 1955) and the density of medium *A* was 1·14. The tube (10 ml.) was centrifuged for 10 min. at 730 *g* in a MSE refrigerated centrifuge ($r_{min.} = 12{\cdot}3$ cm.; $r_{max.} = 20{\cdot}3$ cm.). After this time the top layer, containing almost entirely mitochondria, was sucked off through a pipette bent through 90° at the tip, and discarded; the middle layer, containing mostly nuclei, was sucked off carefully with a filter pump through a fine glass tube bent through 90° at the tip, and was relayered over 4 ml. of ice-cold medium *A*, and recentrifuged for 20 min. at 2240 *g*. The last layering and centrifuging operation was, if necessary, repeated once. This gave a mitochondria-free preparation containing about 50% of the total nuclei of the homogenate, in which there was 8–9% contamination by whole cells or cell fragments (Fig. 2).

Preservation of nuclei by deep-freezing. The homogenates or isolated nuclei were put into Pyrex test tubes (10 mm. × 75 mm.) closed by rubber bungs. Each tube was sealed by inverting its upper end in acetone–CO_2 at −79° until the thin film of medium round the rubber bung was frozen. It was then lowered into the same freezing mixture and was completely frozen in a few seconds. When it was required to warm up the nuclei or homogenates, the tubes were rotated carefully under the cold-water tap.

EXPERIMENTS AND RESULTS

Expt. 1. *Factors affecting granularity of nuclei*

As shown by Anderson & Wilbur (1952), ionic strength, the concentration of bivalent cation and the pH are all factors affecting the production of granular nuclei by precipitation of nucleohistone. These three effects are related. Our exploration of this complex interrelation is not yet complete, but the two experiments described below have a bearing on the question.

pH *and ionic strength, without added bivalent cation.* The relation between pH and the ionic strength required to cause nuclear granularity was investigated by the technique of perfusion of isolated nuclei on the microscope slide. The solutions all contained 0·29 M sucrose and 0·02 M potassium phosphate buffer at various pH values between 6·0 and 7·2, and KCl at various ionic strengths between 0·03 and 1·03. In each set of experiments a single nucleus was observed and

photographed at a given pH. It was perfused by sucking solutions of increasing ionic strength underneath the coverslip with lens tissue, and photographed at increments of ionic strength of about 0·02. The resulting photographs could be used as points on the figure relating pH and ionic strength, and in this way appearances at different pH values could be conveniently compared. The results showed that with KCl, as also with NaCl, potassium acetate and the potassium salts of ethylenediaminetetraacetic acid (EDTA) and nitrilotriacetic acid, the critical ionic strength above which nuclei are granular (in the absence of added bivalent cation) is about 0·1 at pH 6·8. At pH 6·2 it may be as low as 0·06 and at pH 6·0 as low as 0·03. At all pH values the maximum granularity occurs at $I \simeq 0{\cdot}2$, and as their granularity increases the nuclei may also become irregular and distorted in shape, especially at low pH values. At higher ionic strengths the granularity decreases again, and the normal shape of the nuclei is restored, until at $I = 0{\cdot}5$–$1{\cdot}0$ they are normal in shape and only faintly granular; however, by then they are practically empty, since at this ionic strength both globulins and nucleohistone are soluble. Fig. 3 shows a set of photographs of a nucleus at pH 7·0 between ionic strengths 0·03 and 1·03.

pH *and bivalent-cation concentration at constant ionic strength.* As might be expected, the bivalent-cation concentration required to produce granular nuclei is dependent on pH at a given ionic strength. For example, in a liver homogenate in sucrose buffered with triethanolamine–HCl ($I = 0{\cdot}046$) nuclei were granular below pH 6·25, without the addition of bivalent cation. In addition, the nucleoli showed up well below pH 6·65. This suggested that there was enough bivalent cation present in the homogenate to produce these effects. If the homogenate was first washed twice with 0·3 M sucrose and 0·04 M potassium EDTA at pH 6·9, no granularity could be produced at pH values down to 6·0 without the addition of bivalent cation, and the nucleoli were invisible over this range. At pH 6·0, 0·001 M-$CaCl_2$ was needed to produce granularity in EDTA-washed nuclei, and at pH 7·0 0·002 M-$CaCl_2$ was needed. Experiments with potassium nitrilotriacetate as a complex-forming agent to buffer the bivalent cation will be published later.

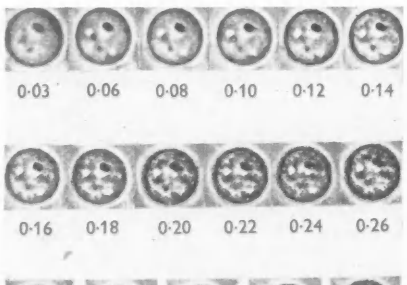

Fig. 3. A nucleus photographed at different total ionic strengths at pH 7·0. The appropriate ionic strength is given under each photograph. Composition of medium: 0·02 M potassium phosphate buffer, 0·29 M sucrose, plus KCl to give varying total ionic strength.

Expt. 2. Factors affecting swelling of nucleus and appearance of nucleoli

The effect of various factors on nuclear size has been investigated by Anderson & Wilbur (1952). They observed that nuclei perfused with sucrose solutions swelled and burst, and suggested that sucrose by itself could be used successfully in isolation media only because of the presence of traces of salts. This suggestion was borne out in the present work, where washing of nuclei with a 0·29 M sucrose–0·04 M EDTA mixture was used as a method of freeing nuclei from bivalent cations. If these EDTA-washed nuclei were then washed with sucrose, they were not only swollen but surrounded by 'haloes' of nucleohistone dissolving out of the nucleus because of the low ionic strength (Lilienfeld, 1894; Stern, Goldstein, Wagman & Schryver, 1947). It is therefore likely that swelling and clumping together of nuclei in media of low ionic strength (Fig. 4) is the first stage in extraction of nucleohistone from the nucleus, and that halo formation (Fig. 5) is the second stage, which takes place only when all the bivalent cations have been removed. Another effect of low ionic strength and absence of bivalent cations is disappearance of nucleoli, as

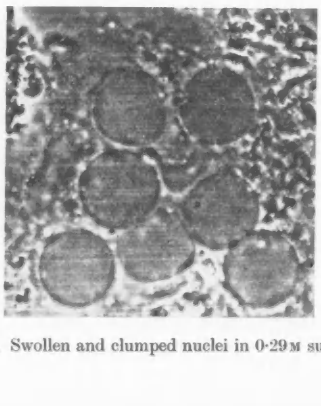

Fig. 4. Swollen and clumped nuclei in 0·29 M sucrose.

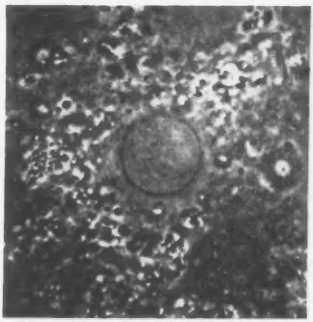

Fig. 5. Halo around nucleus in 0·29 M sucrose, after two washings with 0·29 M sucrose plus 0·04 M ethylenediaminetetraacetic acid.

mentioned under Expt. 1. Nucleoli appear normal, in the absence of any other ions, at a bivalent-cation concentration above about 0·0005 M, at pH 6·9; Ca^{2+}, Mg^{2+} and Ba^{2+} are equally effective. In the absence of bivalent cations the ionic strength has to be higher for preservation of nucleoli; $I \simeq 0{\cdot}08$ gives a normal appearance. It is therefore apparent that the conditions for preservation of nucleoli are similar to those for production of granularity, the limit of bivalent-cation concentration or ionic strength being slightly lower for nucleolar preservation; both conditions are affected in the same way by pH.

Expt. 3. *Factors affecting clumping of nuclei and mitochondria*

Generally speaking, conditions such as a high ionic strength or bivalent-cation concentration which prevent nuclear clumping encourage mitochondria to clump. This makes separation of nuclei from mitochondria difficult, and since clumping of mitochondria may damage them and allow them to release enzymes harmful to nuclei (see Expt. 6) it should also be avoided for this reason. In addition, the concentration of bivalent cation necessary to prevent nuclear clumping is very near to that which causes nuclear granularity. However, if 60 % (v/v) glycerol, 25 % acetone or 14 % dioxan is added, nuclear clumping can be prevented without addition of bivalent cation. Since these solutions have similar fairly low dielectric constants [60, 67 and 67 at 25° compared with 78 for water; Albright (1937), Critchfield, Gibson & Hall (1953)] their anticlumping effect is possibly due to a change in the ion atmosphere preventing incipient dissociation and accompanying stickiness of nucleohistone. The change in dielectric constant alters the effect of ionic strength on granularity studied above, but quantitative data on this point are not yet available. It was found that glycerol was the most suitable of these three substances, because acetone and dioxan slowly caused DNA to become non-extractable. Glycerol was harmless to nuclei at all concentrations tried, i.e. up to 70 %; 60 and 70 % glycerol (cf. Schneider, 1955; Dounce, 1955) also preserved the extractability of nucleohistone from nuclei in a homogenate left for several hours at room temperature, even in the absence of sucrose or glycerophosphate (see Expt. 6), possibly by inhibiting deoxyribonuclease (DNAase). However, the density and viscosity of these high concentrations of glycerol make them impracticable for routine centrifugal sedimentation of nuclei. It was found that 40 % glycerol was effective in preventing nuclei from clumping provided that a low concentration of bivalent cation was also present. For example, 40 % glycerol and 0·001 M-$MgCl_2$ were used in medium *A*, and if either substance was omitted clumping of nuclei resulted (Figs. 6, 7). Besides preventing nuclear

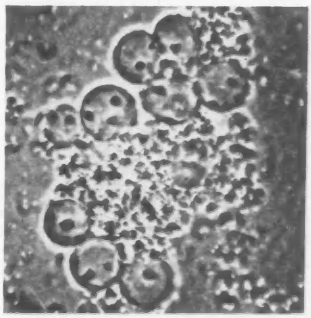

Fig. 6. Clumped nuclei in medium *A* with glycerol omitted.

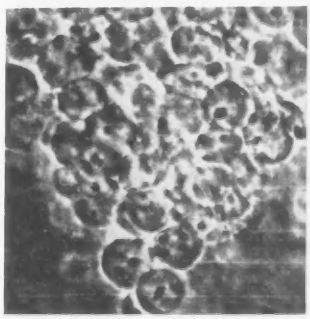

Fig. 7. Clumped nuclei in medium *A* with $MgCl_2$ omitted.

clumping, 40% glycerol prevented contamination of nuclei by mitochondria during centrifugal separation, partly by preventing mitochondrial clumping, and partly by providing, in the layering procedure, a suitable density gradient. A further advantage resulting from the presence of glycerol in the medium was that homogenates and purified nuclei could be deep-frozen at −79° for several weeks without apparent damage (Fig. 8). Dallam (1955) has also described a method for centrifugal separation of nuclei and cytoplasmic granules with high concentrations of glycerol.

Expt. 4. *Factors affecting bleb formation on nuclei*

Anderson (1953*a*) showed that the formation of blebs or bubbles on the surface of nuclei in distilled water or various concentrations of salts could be prevented by the presence of concentrations of sucrose of 0·05 M and above. The fact that this bleb formation occurred in isolated nuclei suggests that it was a non-enzymic phenomenon, or at any rate was not due to the liberation of degradative enzymes from mitochondria. However, Anderson (1953*b*) has shown also that bleb formation is increased in nuclei which have previously been treated with DNAase, and may then occur even in isotonic media. In the present experiments blebs were formed on nuclei left in media of quite high ionic strength in the presence of mitochondria, provided that neither sucrose nor glycerophosphate was present (Fig. 9). They were often accompanied by a loss of density of the nucleus, which has been shown by Anderson (1953*b*) to be a result of DNAase action (see Expt. 6).

Fig. 8. Nuclei in medium *A*, after deep-freezing at −79° for 3 weeks.

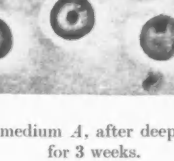

Fig. 9. Bleb formation by nucleus in medium containing 0·06 M-NaCl, 0·005 M potassium phosphate buffer (pH 7·0) and 40% glycerol.

Expt. 5. *Factors affecting extractability of DNA from nuclei*

If the nucleoprotein of the nucleus has been degraded by the action of mitochondrial enzymes, or by non-enzymic means, it will no longer be extractable from the nucleus by M-NaCl or -KCl as a viscous gel forming a 'halo' round the nucleus. This appears to be a very sensitive test for degradation, more so than the macroscopic test of gel formation (Dounce, 1955; Dounce & Monty, 1955) and one which is easily applied both to free nuclei if there are surrounding mitochondria to show the halo up, and to nuclei still inside cells, which behave in the same way as free nuclei (Fig. 10). The halo round free nuclei can also be shown up, in the absence of mitochondria, by methyl-violet staining (Fig. 19). In this work the test of halo formation was used to check the suitability of various suspension media for nuclei. The following solutions preserved the extractability of nucleohistone in the presence of mitochondria at pH 6·9 for some hours.

Sucrose; glycerophosphate. Sucrose or glycerophosphate or both (see Expt. 6), probably acting by preventing liberation of hydrolytic enzymes from mitochondria.

Glycerol. Glycerol (70%, w/v) (see Expt. 3), probably acting by inhibiting hydrolytic enzymes.

0·2 M-NaCl *or* 0·2 M-KCl. These were tried because of the finding by Webb (1953) that 0·2 M-NaCl inhibited the DNAases of calf thymus and leukaemic mouse tissue. Both of course made the nuclei granular, but this granularity was reversible on washing, and good haloes were subsequently formed on addition of M-NaCl.

0·06 M-$MgSO_4$. This was tried for the same reason, and worked about as well as NaCl and KCl; it also made nuclei reversibly granular.

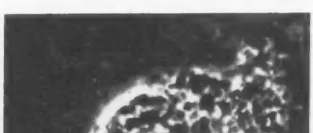

Fig. 10. Halo around nucleus inside cell, formed by suspending freshly prepared homogenate in medium *A* in M-NaCl.

0·01 M-$ZnSO_4$. This was also mentioned by Webb as an inhibitor of DNAases. It was rather less effective than the other inhibitors.

These findings suggest that in the absence of sucrose or glycerophosphate any substance which inhibits DNAase without at the same time denaturing nucleohistone will protect the nucleohistone while the mitochondria are being removed. However, 70 % glycerol is troublesome as a medium for centrifugal separation, and 0·2 M-NaCl or -KCl would be expected to extract globulins from nuclei; these have been shown (Kirkham & Thomas, 1953) to constitute one-third to one-half of the dry weight of the nuclei. It is also not certain that nuclei which have been made granular by any means are completely undamaged, even if the granularity is reversible. It is therefore probable that the simplest and most convenient method of preserving nucleohistone is to keep the mitochondria intact by means of sucrose or glycerophosphate while they are being removed.

Various substances besides M-NaCl or M-KCl will extract DNA from intact nuclei, and will produce haloes round the nucleus. These include heparin (Anderson & Wilbur, 1951), 0·05 M sodium hexametaphosphate, 0·03 M potassium triphosphate ($K_5P_3O_{10}$), 0·03 M adenosine triphosphate, 0·01 M potassium polymethacrylate, 0·06 M sodium barbitone buffer, 0·3 M triethanolamine–HCl buffer, 0·3 M tetraethylammonium chloride and 0·2 M tetramethylammonium chloride. (The concentrations given are the lowest needed to produce clear and definite haloes at pH 6·9.) The first five substances probably act by displacing nucleic acid from histone.

If a solution of histone itself (calf-thymus histone, supplied by Dr P. W. Kent) is added to a homogenate in sucrose, a characteristic type of peripheral and irregular granularity of the nucleus appears (Fig. 11). This happens at quite low concentrations of histone (0·14 %, containing about 0·003 M amino groups). It is probably due to precipitation of the histone with nucleic acid on the nuclear membrane, and may be related to the Liesegang-ring phenomenon, depending on the counter-diffusion of DNA and histone. This was investigated in experiments in which 0·04 M potassium triphosphate was added to a homogenate, together with varying amounts of histone. When the histone was equivalent to 0·003 M amino groups, haloes were formed as usual by the triphosphate, but there was no peripheral granularity. In this case presumably there was no region in which the counter-diffusion of DNA and histone allowed the solubility product of nucleohistone to be reached. When the equivalent concentration of histone was 0·015 M, both haloes and peripheral granularity appeared, suggesting that the solubility product of nucleohistone was reached at the nuclear membrane but not outside it, although some DNA was still diffusing through. If the histone concentration was increased still further, there was peripheral granularity but no halo formation, presumably because of complete precipitation of DNA by histone at the membrane. If 0·2 % protamine sulphate (British Drug Houses Ltd.) was added to a nuclear preparation by perfusion under the coverslip, the nuclei shrank and became extremely refractive. A fine granularity appeared all through the nucleus, which later condensed into large lumps (Fig. 12). Since protamine has a smaller molecular weight than histone, it diffuses further into the nucleus before precipitating with DNA.

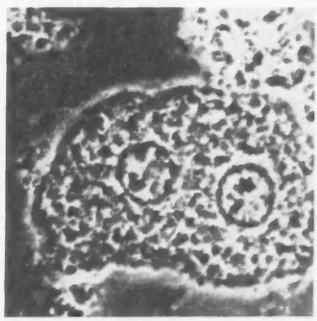

Fig. 11. Peripheral granularity of nuclei (inside a binucleate cell), produced by addition of 0·14 % calf-thymus histone to homogenate in sucrose plus potassium phosphate buffer (pH 6·8).

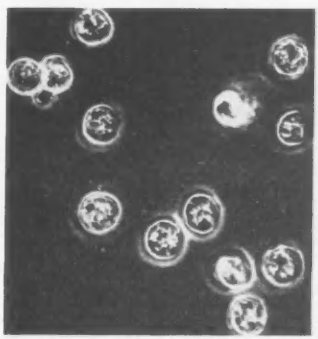

Fig. 12. Internal granularity of nuclei, produced by perfusion on a slide with 0·2 % protamine sulphate. (Nuclei were prepared in 0·115 M potassium glycerophosphate, 40 % glycerol, 0·3 M sucrose and 0·001 M-$MgCl_2$.)

Expt. 6. Further studies of media containing potassium glycerophosphate

The work of Appelmans & Duve (1955) on 'osmotic activation' of mitochondria, and its prevention by sucrose and potassium glycerophosphate, led to the hypothesis that sucrose protected nuclei in a homogenate from enzymic bleb formation and emptying by preventing liberation of harmful enzymes, probably DNAase from mitochondria. Potassium glycerophosphate was therefore tried as a whole or partial substitute for sucrose, and was found to be even more effective than iso-osmolar concentrations of sucrose in protecting nuclei (Philpot & Stanier, 1955). For example, 0·091M or 0·115M potassium glycerophosphate (osmotically equivalent to 0·24M and 0·3M sucrose respectively) preserved nuclei equally well, whether or not sucrose was also present, but the higher concentration was the more effective; concentrations higher than 0·115M were no better. 0·065M Potassium glycerophosphate (osmotically equivalent to 0·17M sucrose) preserved nuclei better than 0·3M sucrose, but the combination of 0·065M potassium glycerophosphate and 0·3M sucrose proved better than these concentrations of either substance alone, so here the effect seemed to be additive.

Effect of amount of mitochondrial contamination

The effect of mitochondrial contamination in hastening emptying of nuclei was shown by isolating nuclei in the complete protective medium (0·115M potassium glycerophosphate, 0·3M sucrose, 40% glycerol, pH 7·0), and then resuspending them, with or without the addition of mitochondria, in either the same medium or the same medium without potassium glycerophosphate; the latter medium contained 0·01M potassium phosphate buffer, pH 7·0. All four preparations were left at room temperature for 25 hr., and then photographed.

Figs. 13 and 14 show nuclei from preparations to which mitochondria had not been added. The nuclei from the medium containing potassium glycerophosphate are full (Fig. 13), and those from the medium without potassium glycerophosphate almost equally so (Fig. 14). Figs. 15 and 16 show nuclei from preparations in which mitochondria were present. When potassium glycerophosphate was present the nuclei again remained full (Fig. 15), but when it was absent they became empty (Fig. 16), even though sucrose was present.

The fact that the protective action of potassium glycerophosphate on nuclei is seen only or mainly in the presence of mitochondria supports the theory that it acts by protecting nuclei from mitochondrial enzymes. However, nuclei among mitochondria eventually become empty even in the presence of potassium glycerophosphate, and the number of mitochondria present will determine how soon this happens. For example, 0·115M potassium glycerophosphate (with or without sucrose) prevented nuclei from emptying for 46 hr. at room temperature in the presence of mitochondria, but for up to 100 hr. in a preparation which had been partially freed of mitochondria by two centrifugings. The nuclei retained their 'full' appearance and their ability to form haloes on addition of M-NaCl (Figs. 17–19). The only difference seen in the preparations without sucrose was possibly a greater tendency to bleb formation; even in the presence of sucrose there was some bleb formation after 20 hr. at room temperature.

Fig. 13

Fig. 14

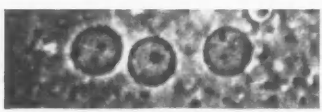

Fig. 15

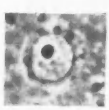

Fig. 16

Figs. 13–16. Nuclei after 25 hr. at room temperature in: Fig. 13: complete medium (0·115M potassium glycerophosphate, 40% glycerol, 0·3M sucrose) without mitochondria; Fig. 14: without potassium glycerophosphate medium and without mitochondria; Fig. 15: complete medium with mitochondria; Fig. 16: medium without potassium glycerophosphate and with mitochondria.

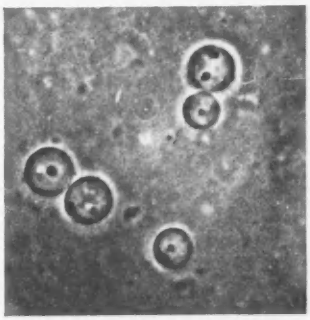

Fig. 17. Nuclei after standing at room temperature for 73 hr. in medium containing 0·115 M potassium glycerophosphate, 40% glycerol and 0·0006 M-$MgCl_2$.

Fig. 18. Haloes around nuclei inside cells, formed by suspending in M-NaCl a homogenate which had stood at room temperature for 67 hr. in 0·115 M potassium glycerophosphate, 40% glycerol, 0·3 M sucrose and 0·0002 M-$MgCl_2$.

Fig. 19. Methyl-violet-stained halo around free nucleus. Halo was formed by suspending in M-NaCl a nuclear preparation which had stood at room temperature for 96 hr. in 0·115 M potassium glycerophosphate, 40% glycerol, 0·3 M sucrose and 0·001 M-$MgCl_2$; aqueous methyl violet was run in under the coverslip.

Comparison of potassium glycerophosphate with potassium phosphate and other buffers

To investigate the possibility that potassium glycerophosphate was preserving nuclei simply by acting as a pH buffer, its effectiveness was compared with that of lutidine, barbitone and triethanolamine buffers at pH 6·9: 0·3 M sucrose and 40% glycerol were the other components of the system. With all three buffers nuclei started to look empty and DNA became non-extractable in a viscous form after the homogenate had stood for an hour or two at room temperature. The possibility was tested that potassium glycerophosphate was working by virtue of its osmolarity or its ionic strength or both. Accordingly, a system was tested which contained K_2SO_4, lutidine and sucrose and had the same osmolarity, ionic strength and buffering power as 0·065 M potassium glycerophosphate and 0·3 M sucrose. It proved to be no more effective than the simple buffer systems. On the other hand, potassium phosphate buffer (pH 6·9), used at concentrations much higher than that needed for pH buffering, appeared at first sight to be almost as effective as potassium glycerophosphate, especially when sucrose was present; the concentration needed for full effect (0·125 M) was a little higher than that of potassium glycerophosphate. However, long-term experiments showed that it was considerably less effective, and was much more sensitive than potassium glycerophosphate to the absence of sucrose. In a typical experiment, liver homogenates in the solutions under test were washed by centrifuging for 15 min. at 25 000 *g*, followed by resuspension in the same solution. They were then allowed to stand at room temperature, and were examined at intervals. After 46 hr. the preparation in 0·125 M potassium phosphate and 40% glycerol contained few full nuclei, many blebs, and only a few nuclei which formed haloes on suspension in M-NaCl. If 0·3 M sucrose was present as well, there were more full nuclei and fewer blebs after this time, and haloes were more consistently formed. The preparation in 0·115 M potassium glycerophosphate and 40% glycerol contained full nuclei, some of which had formed blebs, and good haloes were produced on addition of M-NaCl. The preparation containing 0·3 M sucrose in addition showed no improvement on this, except that only very occasional blebs were seen.

Effect of glycerol on emptying

In all these solutions, 40% glycerol was used to prevent clumping of nuclei, and in addition it appeared to have a convenient bacteriostatic action. A previous experiment had shown that the ability of a preparation to form 'haloes' is not altered by the presence of 40% glycerol.

Effect of $MgCl_2$ *and* $CaCl_2$ *on emptying*

0·115M Potassium glycerophosphate apparently provides a sufficiently high ionic strength to prevent nuclei from clumping, provided that 40% glycerol is also present. In the experiment described above, in which the nuclei were unclumped after centrifuging in 0·115M potassium glycerophosphate and 40% glycerol, $MgCl_2$ was present at various concentrations between zero and 0·04M to test its effect on nuclear emptying. The concentration of $MgCl_2$ appeared to have little effect on the preservation of the nuclei, though there was a suggestion that the nuclei, which started by being very granular (at $MgCl_2$ concentrations of 0·005M and above), formed less consistently good haloes later on, even though this granularity gradually decreased or disappeared. On the other hand, 0·001M-$CaCl_2$ was shown to hasten the emptying of nuclei in a homogenate; this effect was accompanied by a characteristic 'fluffy' appearance of the mitochondria, even in the presence of 0·3M sucrose.

The experiments described in Expt. 6 are consistent with the theory that sucrose and potassium glycerophosphate protect nuclei by preventing damage to mitochondria and their consequent liberation of harmful enzymes. This enzymic explanation was given additional support by the demonstration of Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) that DNAase, ribonuclease and cathepsin are among the enzymes associated with 'lysosomes', the mitochondrial granules rich in hydrolytic enzymes, from which they are liberated by 'osmotic activation'. The present results, and the experiments on inhibitors mentioned under Expt. 5 are consistent with the enzyme concerned being DNAase; although Dounce, Witter, Monty, Pate & Cottone (1955) and Dounce & Monty (1955) who have reached the same conclusion about the importance of protecting mitochondria if the gel-forming power of nuclei is to be preserved, consider that there is some evidence against this.

DISCUSSION

Some of the rules for the design of a nuclear medium given above, which are largely empirical, can be explained in terms of the concept of the nucleus that has emerged from the work of many investigators but which is still to some extent controversial. One form of this concept may be stated as follows.

The normal resting nucleus is an optically homogeneous nucleohistone gel enclosed in a coarsely permeable membrane and containing a few denser structures, the nucleoli. Its submicroscopic interstices contain various 'euglobulins' (i.e. proteins insoluble at low ionic strength), some of them enzymes, which are in gel form but which can be dissolved by ionic strengths above about 0·1, or higher if the dielectric constant is lowered by glycerol. The cell sap probably penetrates freely through the nuclear membrane, and its components may reach into the interstices of the gel to an extent probably dependent on their molecular weights. Whether this penetrating cell sap is part of the nucleus is a purely linguistic question which is nevertheless of importance because it probably underlies the controversy between the advocates of wet and dry methods of isolating nuclei. The nucleohistone gel has a net negative charge (cf. Philpot & Stanier, 1954) which would be expected to give it electro-osmotic properties. It therefore swells and even dissolves as the ionic strength is lowered (Stern *et al.* 1947), provided that bivalent cations are absent, while at higher ionic strength (about 0·14) it precipitates, making the nucleus granular, and at still higher ionic strength it dissolves by dissociation of the histone from the nucleic acid (Mirsky & Pollister, 1946). Possibly also the spare negative charges of the DNA can form dissociable salt-linkages with the non-histone protein, and may be partly or wholly responsible for the euglobulin properties of the latter (cf. Dounce, 1952). The granularity produced by change of ionic strength, pH or bivalent-ion concentration, or the visibly different type produced by histone, may be related to the condensation of chromosomes at mitosis, but is less regularly arranged. Possibly a better resemblance could be produced by slower precipitation.

There is thus a wide choice of media that can keep the nucleohistone and the globulins in their insoluble-gel form; but it is also necessary during isolation of nuclei to keep the mitochondria or rather the 'lysosomes' (Duve *et al.* 1955) in good condition so that they do not liberate lytic enzymes such as nucleases and cathepsin, which can cause rapid emptying of nuclei and also bleb formation (Anderson, 1953*b*). This probably accounts partly or wholly for the beneficial effect of glycerophosphate and sucrose. We suspect that glycerophosphate acts mainly by preventing some degradation inside the lysosome, leading to low-molecular breakdown products with a high intrasomal osmotic pressure, which can be balanced by external sucrose to prevent bursting. If this is true, metabolically normal lysosomes would need less sucrose, which may explain the absence of sucrose or of any obvious substitute in natural cell sap, and which may also explain why glycerophosphate can prevent nuclear emptying at lower osmolarity than is needed with sucrose.

SUMMARY

1. For isolation of rat-liver nuclei a medium is recommended having this composition: 0·3 M sucrose, 0·065 M potassium glycerophosphate, 0·001 M magnesium chloride, 40 % glycerol; pH 7·0.

2. Phase-contrast photographs are shown of nuclei in this medium, and in modifications of it causing various abnormal appearances.

3. Nuclei in the recommended medium retain their normal appearance for at least 6 hr. at room temperature, after which they are still able to form haloes of soluble viscous nucleic acid when transferred to molar sodium chloride.

4. If the glycerophosphate concentration is increased to 0·115 M, with or without magnesium chloride, nuclei retain their normal appearance for 100 hr. at room temperature, apart from slight clumping; but the higher ionic strength of this medium may sometimes be a disadvantage.

We are grateful to the Bersworth Chemical Co. for the gifts of ethylenediaminetetraacetic and nitrilotriacetic acids, to Dr P. W. Kent for a gift of calf-thymus histone and to Miss B. Hunt, Miss M. Tobias and Mr R. Gutsell for technical assistance.

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Cerebral Diphosphoinositide Breakdown: Activation, Complexity and Distribution in Animal (mainly Nervous) Tissues

By R. RODNIGHT
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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Suspensions of guinea-pig brain rapidly attack the 'inositol phosphatide' fraction of ox-brain kephalin, liberating into the medium acid-soluble inositol and phosphate (Sloane-Stanley, 1953). Presumably cerebral tissue contains enzymes catalysing the breakdown of the diphosphoinositide (DPI), shown by Folch (1942) to be an important constituent of brain kephalin. The interesting feature of this reaction is its rate, which is outstandingly higher than the rates previously reported for the *in vitro* hydrolysis of other brain lipids (Sloane-Stanley, 1952). On a molar basis it is comparable to the rate of oxygen uptake by cerebral preparations.

The present work is mainly concerned with the distribution of the enzyme system and its sensitivity to certain cations and drugs. For most experiments a saline suspension of the 'inositol phosphatide' fraction of kephalin has been used as substrate, but preliminary observations on the breakdown of the water-soluble potassium salt of cerebral DPI (Folch, 1949*a*) are also included.

EXPERIMENTAL

Enzyme activity was studied in two ways. (*a*) By following acid formation manometrically in bicarbonate-buffered saline solutions (Sloane-Stanley, 1953). The rate of CO_2

evolution during the first 15 min. was taken as a measure of the enzyme content of the tissue. Total P rendered acid-soluble was determined at the end of the experiment and in some cases inorganic P was also determined. (*b*) By determining phosphates (and in one experiment inositol) at various times during the course of the reaction.

Substrates

'*Inositol phosphatide.*' This was prepared from ox brain (from the slaughter house) or post-mortem human brain by the procedure of Folch (1942). Some samples (Table 1) were further fractionated with chloroform–methanol (Folch, 1949*b*). After dialysis the aqueous lipid emulsion was brought to pH 7 with ice-cold N-KOH before freeze-drying. The dried lipid was stored in a vacuum desiccator over silica gel at 0°.

It was found difficult to reduce the inorganic P content of the samples below about 0·13% by dialysis in bulk. This was done by washing small quantities of the substrate, as required, in the following manner. Lipid (100 mg.) was suspended in 3 ml. of ice-cold glass-distilled water (in an all-glass homogenizer), and centrifuged at 25 000 *g* for 20 min. at 0°. The supernatant, which was usually clear, was discarded and the washing process repeated. The deposit was then dried with acetone (in which case it was used within 2 days), or freeze-dried and stored as described above.

Inositol in the samples was determined and an estimate of their DPI content made by taking its inositol content to be 21% (Folch, 1949*a*) (Table 1). The structure or mol.wt. of DPI is not known, but analysis (Folch, 1949*a*) indicates that each molecule of inositol diphosphate is esterified to a mono- or di-glyceride; the mol.wt. of this would lie between 750 and 950, depending on its fatty acid composition. To assess the proportion of DPI degraded by the enzyme, a figure of 0·8 mg. has been taken as equivalent to 1 μmole.

From previous work (Folch, 1942) the remaining lipids in the samples were assumed to consist of phosphatidyl serine and phosphatidyl ethanolamine, neither of which is attacked by brain suspensions (Sloane-Stanley, 1953).

Potassium diphosphoinositide (Folch, 1949*a*, *b*). This was prepared and generously given by Professor Folch-Pi. It dissolved in water (or 0·9%, w/v, NaCl) to form a clear pale-yellow solution, stable at 4° for at least 5 days. After dialysis for 48 hr. against distilled water it was freeze-dried, stored at 4° in a vacuum desiccator, and immediately before an experiment was redissolved in the appropriate medium.

Table 1. *Inositol and approximate diphospho-inositide contents of samples of crude substrate*

Average of results obtained by yeast-growth method (Sloane-Stanley, 1953) and HIO_4-oxidation method (Platt & Glock, 1943). Approx. DPI content was calculated from the data of Folch (1949*a*): inositol content of purified DPI = 21%.

Source	Inositol (%)	Approx. DPI (%)
Ox brain *A**	9·9	47
B	4·8	23
*C**	6·1	29
D	6·5	31
Human brain*	11·5	55

* Precipitated with chloroform–methanol (after Folch, 1949*b*).

Tissue suspensions

Tissue was obtained as soon as possible after death and suspended in about four times its wet wt. of ice-cold 0·9% (w/v) NaCl in a glass homogenizer. The dry wt. of tissue was determined by heating 1·0 ml. samples of suspension at 110° for 3 hr., allowance being made for added salt. To convert the dry wt. into wet wt., a factor of 5 was used for whole-brain preparations from small laboratory animals; in other cases the dry wt.:fresh wt. ratio was determined by drying a sample at 110°.

Saline suspensions were stable for 2–3 weeks at 4° with respect to the properties of the system being studied, provided that toluene was added. For example, a preparation of whole brain lost less than 4% of its activity in 14 days.

For experiments in which a low initial P was desirable, suspensions were dialysed in cellophan bags for 48 hr. against 0·9% (w/v) NaCl, at 4°; such treatment did not alter enzyme activity.

Laboratory animals were killed by decapitation and bled before removal of the brain. Sheep brain was obtained from the slaughter house about 1 hr. after death and brought to the laboratory packed in ice. Infant-human brain from a child 9 days old, who had died from anoxia, was obtained some 24 hr. after death. Adult-human brain was obtained from neurosurgical operations and brought to the laboratory in ice immediately after removal (for further details see McIlwain, Ayres & Forda, 1952).

The stability of the system in intact brain after death was investigated by control experiments. Guinea-pig brain was removed in the usual manner, one half was immediately suspended in saline and the other left (*a*) at room temp., and (*b*) at 4° for 6 hr. before suspending. No loss of activity could be detected.

Reaction with 'inositol phosphatide'

Acid formation. Small Warburg vessels of conventional pattern but of 4–5 ml. capacity (Barker, 1949) were used to conserve substrate. Tissue preparations were diluted to the desired concentration with a bicarbonate saline at pH 7·4 and freshly equilibrated with $N_2 + CO_2$ (95:5, v/v). The final concentration of constituents in the reaction mixture was: 120 mM-NaCl, 4·7 mM-KCl, 2·5 mM-$CaCl_2$, 1·2 mM-$MgSO_4$, 25 mM-$NaHCO_3$. Substrate was freshly weighed and suspended in the medium with a glass homogenizer. Added compounds were incorporated in the saline used for diluting the tissue suspensions.

In a typical experiment 0·4 ml. of dilute tissue suspension containing about 12–15 mg. of fresh wt. of tissue was pipetted into the main compartment, and 0·1 ml. (5 mg.) of lipid suspension into the side bulb. After gassing with $N_2 + CO_2$ (95:5, v/v) for 10 min., and equilibrating in the thermostat for 15 min., the reaction was started by tipping the flasks and readings were taken every 3 or 5 min. for 30–40 min. Control flasks containing tissue or substrate only were run in parallel and any gas changes allowed for in calculating the rate of CO_2 evolution. Blank values were of the same magnitude as those described by Sloane-Stanley (1953), i.e. sometimes up to 20% of total, but usually between 10 and 15%. Preliminary experiments with varying amounts of substrate established the level required to produce the

maximum initial rate with a constant weight of tissue. The quantity of tissue added was sufficient to produce a smooth evolution of CO_2, and small variations of a few mg. did not significantly alter the initial rate per unit wt. of tissue.

The reaction was stopped by adding 1·0 ml. of ice-cold 0·45 N-$HClO_4$. After centrifuging the supernatant was filtered through 4·5 cm. Whatman no. 542 papers (a few early experiments), or more often through small pads of acid-washed asbestos, supported by cotton wool in the drawn-out stem of a 3 cm. filter funnel. A sample of the filtrate was diluted with sufficient NaOH solution nearly to neutralize the acid present, and the solution was stored at $-20°$ for P estimation. No increase in the inorganic fraction of the total P occurred under these conditions.

Phosphate liberation. In most cases the reaction was run in air in 0·067 M glycylglycine buffer (pH 7·2) diluted from 0·5 M stock solution with a salt solution of the desired composition. Samples were withdrawn at intervals for P estimation. In a few experiments P liberation was followed in bicarbonate saline in manometric apparatus by including several flasks of the same composition and removing them from the thermostat at intervals. No difference in the kinetics of the reaction was observed between experiments run in bicarbonate buffer in N_2+CO_2 mixture and in glycylglycine buffer in air.

For experiments of 60 min. duration or less blank determinations were made by mixing tissue and substrate, and immediately precipitating by adding the 0·45 N-$HClO_4$. With freshly washed substrate and dialysed brain these were never more than 15% of the total P evolved enzymically. For experiments extending over a longer period this method was unsatisfactory, since it was found that although no non-enzymic production of P took place in the substrate when shaken in buffer in the thermostat for several hours, the acid-soluble P content of brain suspensions under these conditions tended to increase (sometimes by 100%), and this invalidated the zero blank. In these cases a separate tissue blank was run and analysed at the end of the experiment; from the difference between this and a zero brain blank, corrections for intermediate times were calculated.

Experiments with potassium diphosphoinositide

Only a limited quantity of this substance was available and consequently the scale of the experiments was reduced to one-quarter of that employed with the cruder substrate. Thus in a typical experiment 0·025 ml. of a solution of the lipid containing 16 mg./ml. was added to 0·1 ml. of brain suspension in a 75 mm. × 9 mm. test tube; after incubation 0·125 ml. of ice-cold N-$HClO_4$ in 0·1 M-$CaCl_2$ was added, followed by 0·125 ml. of 0·95 N-KOH. After centrifuging phosphates were determined in the supernatant fluid; filtration was unnecessary. The addition of Ca ions to the perchloric acid improved the completeness of precipitation of unchanged lipid.

Analytical methods

Phosphates. These were determined by a micro modification of the method of Martin & Doty (1949), omitting the silico-tungstate reagent. Colours were read at 700 mμ. in a Beckman DU Quartz or Hilger Uvispek spectrophotometer. For the determination of total P, organically-bound P was hydrolysed by boiling with 10–11 M-$HClO_4$, and neutral instead of acid ammonium molybdate was used. The recovery of added inorganic P with this procedure was satisfactory.

In experiments with 'inositol phosphatide' the method covered the range 0–6 μg. of P in a final volume of 4 ml. With potassium diphosphoinositide the scale was reduced to estimate 0–2 μg. of P in a volume of 1 ml.

Inositol. Inositol was determined in lipid samples by the yeast-growth method as described by Sloane-Stanley (1953), and by the chemical method (oxidation by HIO_4) of Platt & Glock (1943) as adapted by Sloane-Stanley (1953).

In reaction products the chemical method only was used. A similar procedure to that described by Sloane-Stanley (1953) was used except that the succinate buffer was omitted and filtration effected through asbestos. Good recovery of added inositol was obtained; blank determinations of the amount of inositol originally present and of that produced non-enzymically from both substrate and brain suspensions during the experiment were made (combined value less than 10% of the total liberated).

RESULTS

Activation of the reaction with 'inositol phosphatide' as substrate

Preliminary experiments. Fig. 1*A* illustrates the release of H ions (judged by CO_2 evolution) and phosphates from 'inositol phosphatide' by a whole-

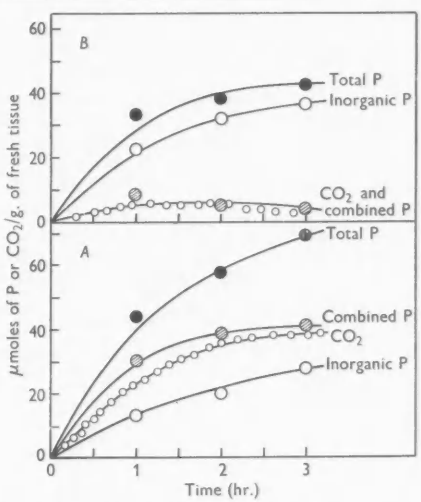

Fig. 1. Release of H ions (CO_2 evolution) and phosphates from 'inositol phosphatide' by a suspension of whole guinea-pig brain in bicarbonate-buffered salines. Conical Warburg vessels, capacity 18–20 ml.; gas phase N_2+CO_2 (95:5, v/v). The curve 'combined acid-soluble P' represents the difference between the curves for total acid-soluble P and inorganic P. *A*, medium contained: 120 mM-NaCl, 4·7 mM-KCl, 2·5 mM-$CaCl_2$, 1·2 mM-$MgSO_4$ and 25 mM-$NaHCO_3$. *B*, only 129 mM-NaCl and 25 mM-$NaHCO_3$ were present. (For reasons of clarity only one line has been drawn in *B* for both CO_2 and combined P.)

brain preparation (guinea pig) in bicarbonate medium containing K, Mg and Ca. Fig. 1*B* illustrates the release of the same ions when the reaction was carried out in a bicarbonate medium of the same tonicity and pH, but with the omission of K, Mg and Ca ions. Clearly the presence in the first medium of one of these ions had activated the release of combined P (total acid-soluble P less inorganic P), and to a less extent had inhibited the release of inorganic P. Other experiments with different samples of substrate and various brain preparations gave essentially the same result.

Nature of activation. This was studied in glycylglycine-buffered media by withdrawing samples of the reaction mixture for P estimation at various times (Table 2). Calcium greatly increased the release of combined P, but Mg, K and Ba were without action in this respect; Mg, Ca and Ba all slightly decreased the release of inorganic P. The possibility that this smaller amount of inorganic P found in the presence of bivalent cations was due to losses incurred in working up the reaction mixtures was excluded by adding inorganic P to media containing Ca in the same and greater concentrations than occurred in the experiments, when satisfactory recovery was obtained.

Action of calcium on release of combined acid-soluble inositol. Sloane-Stanley (1953) reported the release of acid-soluble inositol (85–90 % of which was combined) from 'inositol phosphatide' when incubated with brain suspensions. The influence of Ca on this aspect of the reaction is shown in Table 3. Calcium activated combined-inositol release to the same extent as it did that of combined P; approximately equimolar amounts of each were found in both media at all stages of the reaction studied.

Optimum concentration of calcium for activation. The initial rate of combined P release was greatest in a medium containing mM-$CaCl_2$, but the reaction was maintained longer and the amount of combined P eventually formed was greater in 2 mM-$CaCl_2$ (Fig. 2). Increasing concentrations of Ca also progressively inhibited the release of inorganic P.

The 'inositol phosphatide' used in these experiments contained DPI in the form of its Ca–Mg salt. Analysis of one sample gave: Ca 0·13 %, Mg 1·12 %, figures of the same order as reported by Folch (1942). The question whether this intrinsic Ca was able to activate the enzyme, and might therefore account for the small quantity of combined P released even in a Ca-free medium (Table 2),

Table 2. *Action of calcium, magnesium and barium salts on release of phosphates from 'inositol phosphatide' by brain suspensions*

Whole brain (guinea pig) was suspended in 0·9 % (w/v) NaCl, about 120 mg. of fresh tissue/ml., and dialysed for 48 hr. at 4°. Before an experiment the suspension was diluted to 30 mg./ml. with an isotonic NaCl–glycylglycine medium (pH 7·2), containing $CaCl_2$, $MgSO_4$ or $BaCl_2$ where stated, so that the final concn. of buffer was 0·067 M. A portion (2 ml.) was incubated at 38° with 0·5 ml. of 'inositol phosphatide' (50 mg./ml.). Samples (0·5 ml.) were withdrawn at 0, 30 and 60 min. DPI available was approx. 125 μmoles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

	Total acid-soluble P (μmoles/g. of fresh tissue)		Inorganic P (μmoles/g. of fresh tissue)		Combined P by difference (μmoles/g. of fresh tissue)		Ratio combined P: inorganic P	
Addition to medium	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.	30 min.	60 min.
None	31	36	24	30	7	6	0·3	0·2
3·3 mM-$CaCl_2$	33	51	10	17	23	34	2·3	2·0
2·0 mM-$MgSO_4$	13	23	9	16	4	7	0·5	0·4
3·3 mM-$BaCl_2$	14	18	11	15	3	3	0·3	0·2

Table 3. *Action of calcium on release of combined acid-soluble inositol from 'inositol phosphatide' by brain suspensions*

Whole brain (guinea pig) prepared as in Table 2 and diluted to 43 mg./ml. with NaCl–glycylglycine medium (pH 7·2) containing $CaCl_2$ where stated. A portion (14 ml.) was incubated at 38° with 1 ml. of 'inositol phosphatide' suspension (80 mg./ml.). Samples (3 ml.) were withdrawn at 0, 1, 2 and 4 hr. Total DPI available was approx. 40 μmoles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

	Total acid-soluble P (μmoles/g. of fresh tissue)			Inorganic P (μmoles/g. of fresh tissue)			Combined P by difference (μmoles/g. of fresh tissue)			Combined acid-soluble inositol (μmoles/g. of fresh tissue)		
Addition to medium	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.	1 hr.	2 hr.	4 hr.
None	29	34	39	19	21	21	10	13	18	11	15	18
2 mM-$CaCl_2$	35	43	48	10	14	18	25	29	30	22	25	31

Table 4. *Release of phosphates from potassium diphosphoinositide by brain suspensions*

Whole brain (guinea pig) prepared as in Table 2 and diluted to 30 mg./ml. with NaCl–glycylglycine medium (pH 7·2) containing $CaCl_2$ or $MgSO_4$ where stated. Samples (0·1 ml.) were incubated at 38° with 0·025 ml. of a 'solution' of potassium diphosphoinositide (16 mg./ml.). Total DPI available was approximately 166 μmoles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P. The results quoted for Expts. 2–6 are mean values followed by their S.E.

Expt.	Addition to medium	Total acid-soluble P (μmoles/g. of fresh tissue)		Inorganic P (μmoles/g. of fresh tissue)		Combined P by difference (μmoles/g. of fresh tissue)		Ratio combined P: inorganic P	
		10 min.	30 min.	10 min.	30 min.	10 min.	30 min.	10 min.	30 min.
1 (brain *D*)	None	85	118	29	50	56	68	1·9	1·4
	mM-$CaCl_2$	89	134	17	38	72	96	4·2	2·5
	2 mM-$CaCl_2$	85	128	15	29	80	98	5·3	3·4
	mM-$MgSO_4$	94	122	25	49	69	73	2·8	1·5
	2 mM-$MgSO_4$	95	134	25	50	65	84	2·6	1·7
2–6 (brains *B*, *C* and *D*)	None	70	106	28±0·9	46±2·7	42±4·2	60±5·1	1·5	1·3
	2 mM-$CaCl_2$	78	109	19±1·7	31±1·8	59±6·5	76±7·1	3·1	2·5

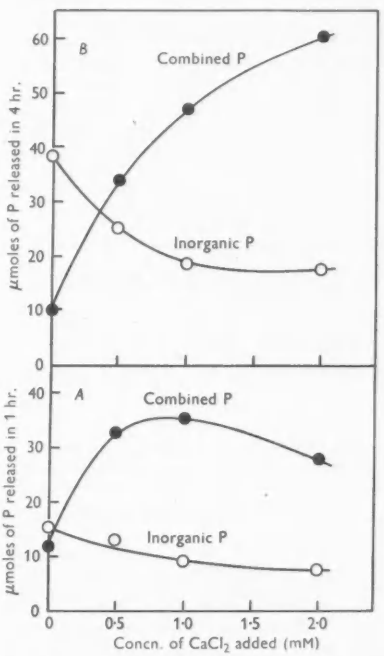

Fig. 2. Action of various concentrations of $CaCl_2$ on release of phosphates from 'inositol phosphatide' by a suspension of whole guinea-pig brain. The reaction mixture was buffered at pH 7·2 with glycylglycine (0·067 M) and contained $CaCl_2$ in the concentrations shown. The curve 'combined acid-soluble P' represents the difference between the curves for total acid-soluble P (not shown here) and inorganic P. *A*, phosphates found after 1 hr.; *B*, phosphates found after 4 hr.

was considered. However, when the reaction was studied in a Ca-free medium containing 10 mM-NaF there was no further reduction in the amount of combined P released, which suggests that this intrinsic Ca was not available.

To investigate the effect of ions on the physical state of the suspended substrate, dilute suspensions of 'inositol phosphatide' were prepared and their turbidity was measured in a Spekker absorptiometer. The addition of either 2 mM-$CaCl_2$ or $MgSO_4$ to suspensions in glycylglycine-buffered isotonic NaCl medium increased their turbidity by about 15%, but microscopical examination of the centrifuged deposit revealed no marked differences in the size of the clumps of lipid particles. The addition of 10 mM-$CaCl_2$ resulted in a much greater turbidity, and flocculation and rapid sedimentation of the lipid occurred on standing.

Experiments with potassium diphosphoinositide

Table 4 summarizes the results of six experiments with whole guinea-pig brain suspensions in which a solution of potassium diphosphoinositide was used as substrate at a final concentration of 3·2 mg./ml., equivalent to approx. 166 μmoles/g. of fresh wt. of tissue. Compared to the reaction with 'inositol phosphatide' the following points are worthy of comment: (*a*) In media containing Ca the proportion of combined P to inorganic P released after incubation for 30 min. was roughly the same with both substrates. (*b*) The breakdown of potassium diphosphoinositide was very rapid, when assessed by the amount of combined P released after 10 min. in media containing Ca. That the rates quoted in Table 4 were still considerably less than the maximum was shown in an experiment in which substrate concentrations of 2, 4 and

6 mg./ml. were employed; the 10 min. total acid-soluble P figures were then respectively 43, 92 and 130 μmoles. This extremely rapid breakdown was confirmed in bicarbonate-buffered saline containing Ca, with a rabbit-brain preparation; at a substrate concentration of 4 mg./ml. the initial rate of CO_2 evolution was 300 μmoles/g. fresh wt. of tissue/hr., compared to figures of 24 and 46 μmoles/hr. obtained with crude substrate and the same species (Table 5). (*c*) An activation by Ca of combined P release from potassium diphosphoinositide could not be unequivocally demonstrated, since the difference between the means quoted in Table 4 for media with and without Ca (at either 10 min. or 30 min.) was only significant to the 0·1 level of probability. On the other hand, as was found with the cruder substrate, Ca significantly inhibited the release of inorganic P ($P < 0{\cdot}01$). A solution of potassium diphosphoinositide in the Ca-free medium was clear and stable; on addition of $CaCl_2$ to 2 mM concentration, a slight turbidity appeared, but no precipitation of the lipid occurred on standing at room temperature.

Distribution of enzyme activity

In experiments described in this section the substrate was 'inositol phosphatide'.

Nervous tissues. A preliminary survey of the distribution of the enzyme system in anatomically defined regions of the central nervous system was made by measuring acid formation, supplemented by total acid-soluble P determinations at the end of the experiments (Table 5). The results suggest that the enzyme activity is fairly uniformly distributed in nervous tissue, although peripheral nerve showed less activity than brain or cord preparations; the higher proportion of connective tissue in nerve may, however, account for this difference. In the human material, adult-brain preparations from biopsy specimens were less active than those from a 9-day old infant brain (*post-mortem*) in which myelination was incomplete.

Further determinations of the rate of phosphate release in glycylglycine media, both with and without Ca, failed to reveal any great variation in activity (Table 6), although the ratio of combined P to inorganic P released tended to be higher with the peripheral nerve and spinal cord preparations. Activation by Ca was demonstrated in all preparations (except spinal cord, which was not tested).

Non-nervous tissues. Suspensions of rat liver, kidney and spleen released H^+ ions and acid-soluble P from the substrate at about the same rate as did cerebral tissues; no activity was shown by either serum or lysed erythrocytes (guinea pig).

When the release of both inorganic and total P was followed some differences in the course of the reaction were found (Table 6). With kidney (either in the presence or absence of Ca) the main end-product of the reaction was inorganic P. A skeletal-muscle preparation released inorganic P at a rate approx. 20% of that found with brain, but no combined P. When Ca was omitted from the medium less combined P appeared with all tissues.

Attempts to influence the reaction with drugs and other compounds

The compounds examined were chosen for one of the following reasons: (*a*) for their known action as inhibitors of other enzyme systems; (*b*) for their known action on the central nervous system in the intact animal; (*c*) for the structural similarity of their molecule (or groups) to inositol.

Table 5. *Diphosphoinositide-splitting enzyme activity in nervous tissues from several species*

Tissues were suspended in 0·9% (w/v) NaCl at about 120 mg./ml. and diluted to 30 mg./ml. with a bicarbonate-buffered medium containing NaCl, KCl, $CaCl_2$ and $MgSO_4$ (see text). A portion (0·4 ml.) was incubated at 38° with 0·1 ml. of 'inositol phosphatide' suspension (50 mg./ml.) under manometric conditions in small Warburg vessels; CO_2 evolution was followed for 30–45 min. after adding the lipid suspension from the side bulb.

Species	Source of tissue	Initial rate of hydrolysis (μmoles of CO_2/g. of fresh tissue/hr.)	Total acid-soluble P after 45 min. (μmoles/g. of fresh tissue/hr.)
Guinea pig	Whole brain	50, 52, 62	49, 56, 76
Guinea pig, rat, rabbit, sheep and monkey	Cerebrum,* cerebellum, thalamus, medulla, pons and spinal cord	24–60	37–74
Cat and monkey	Sciatic nerve	14, 19, 20	12, 16
Human (infant 9 days)	Cerebrum,* cerebellum, olivary nucleus, medulla and pons	42–70	60–82
Human adult (3 cases)	Cerebrum*	24, 25	38, 46
	Grey matter (cortex)	22, 36	36, 50
	White matter (cortex)	20, 20	30, 36

* Cerebral cortex together with some underlying white matter.

Table 6. *Release of phosphates from 'inositol phosphatide' by suspensions of nervous and non-nervous tissues: action of calcium salts*

Tissues were prepared and their enzyme activity was determined as described in Table 2. The DPI available in the substrate was approx. 200 μmoles/g. of fresh tissue (see text). Combined P = total acid-soluble P less inorganic P.

Species	Source of tissue	Addition to medium (2 mM)	Inorganic P (μmoles/g. of fresh tissue)		Combined P by difference (μmoles/g. of fresh tissue)	
			25 min.	60 min.	25 min.	60 min.
Sheep	Cortex	None	26	42	15	15
	Cortex	$CaCl_2$	16	26	37	38
	Subcortical white matter	None	21	31	11	10
	Subcortical white matter	$CaCl_2$	14	26	36	38
Rabbit	Cortex	None	20	31	7	7
	Cortex	$CaCl_2$	10	16	30	45
	Brain stem	None	27	39	3	11
	Brain stem	$CaCl_2$	15	25	36	45
	Sciatic nerve	None	2	6	2	4
	Sciatic nerve	$CaCl_2$	2	5	18	20
Monkey	Spinal cord	$CaCl_2$	5	10	46	57
			20 min.	60 min.	20 min.	60 min.
Guinea pig	Liver	None	6	16	4	14
		$CaCl_2$	5	10	31	44
	Kidney	None	17	28	0	0
		$CaCl_2$	27	36	6	9
	Spleen	None	10	26	31	38
		$CaCl_2$	8	16	45	50
	Skeletal muscle	$CaCl_2$	4	7	1	0

Table 7. *Action of added compounds on breakdown of diphosphoinositide by brain preparations*

Whole brain (guinea pig) was prepared and its enzyme activity determined in the presence and absence of the compounds as described in Table 5; compounds were incorporated in the saline medium used for diluting the tissue suspension. Values quoted are (*a*) the initial rate of hydrolysis (μmoles of CO_2/g. of fresh tissue/hr.) in the presence of compound, expressed as a percentage of the rate in its absence, and (*b*) the total and inorganic P released in 30 min. similarly expressed.

Compound added to medium	Rate of hydrolysis (%)	Total acid-soluble P (%)	Inorganic P (%)
NaCN, 10 mM; 2:4-dinitrophenol, 10 mM; Na L-glutamate, 10 mM	100–123	100–120	83–104
Na iodoacetate, 10 mM	72	83	90
NaF, 10 mM	77	85	125
Glyceryl *o*-tolyl ether (Myanesin), 6·6 mM, 13 mM; mescaline, 30 mM; chloral hydrate, 10 mM; diethylbarbitone, 10 mM; diallylbarbitone, 10 mM	89–108	77–125	78–101*
β-Diethylaminoethyl *p*-aminobenzoate, HCl (Procaine), 10 mM	130	138	160
Streptomycin, 2·5 mM, 10 mM; 4-*n*-hexylresorcinol, 2 mM; inositol, 3·3 mM	91–93	96–115	98–100
γ-Hexachloro*cyclo*hexane (Gammexane), about 1 mM	83	63	82

* Values for Myanesin, mescaline and dial not available.

None of the compounds showed any marked action (Table 7); such changes as were observed were either within experimental error or too small to warrant further investigation considering their high concentrations in the medium.

DISCUSSION

Complexity of the reaction between 'inositol phosphatide' and cerebral tissues. This is illustrated by its time course in media with and without Ca. In the presence of appropriate concentrations of Ca ions, the concentration of acid-soluble organically-combined P, inositol and H^+ ions is increased by 80%, whereas that of inorganic P is reduced by about 40% (Fig. 1 and Table 3).

These observations tend to confirm the suggestion of Sloane-Stanley (1953) that there are at least two enzymes in brain concerned in the breakdown of

the diphosphoinositide in 'inositol phosphatide'; furthermore, they suggest that one of these enzymes is a phosphodiesterase releasing inositol monophosphate, and another a phosphomonoesterase releasing inorganic P. The two reactions appear to occur concurrently, since the present evidence renders it unlikely that the first product of the reaction is inositol metadiphosphate (which is a product of the acid hydrolysis of DPI: Folch, 1949*a*), from which another molecule of P is then split by a second enzyme. If this were the case, the reduction in the amount of inositol monophosphate released in the Ca-free medium should be accompanied by a corresponding reduction in inorganic P; in fact, in the absence of added Ca the inorganic P released is always greater in amount (in some experiments by as much as three molar equivalents) than the acid-soluble inositol.

Nature of calcium activation. The diphosphoinositide present in 'inositol phosphatide' exists as its practically insoluble Ca–Mg salt (Folch, 1942, 1949*a*, *b*), but it is not clear whether the particles of lipid suspended in the medium unite directly with the enzyme, or whether solution occurs first. Certainly the physical state of the substrate is likely to be an important factor influencing the reaction, and it is one which might well be altered by the ionic composition of the medium. Physiological concentrations of Ca are known to modify the interfacial tension in oil–water systems (Netter, 1953, for example).

The possibility cannot be excluded, therefore, that Ca activates the reaction by changing the physical properties of the suspended-lipid particles. If this were the case, however, other bivalent cations might be expected to have a similar action; in fact, although both Ca and Mg increase the turbidity of substrate suspensions only Ca has been found to activate the reaction.

The activation of certain enzymes by Ca is well known. Examples in cerebral tissues are thiamine pyrophosphatase and acid phosphomonoesterase (Pratt, 1954), although both these are also activated by Mg. Calcium has also been implicated in enzymes hydrolysing other lipids: its activation of the hydrolysis of neutral fats by lipases has long been known and has recently been re-examined (Schønheyder & Volquartz, 1945), as has its similar action on snake-venom lecithinase (Holden, 1934; Braganca & Quastel, 1953). Of possibly greater relevance to this work is the lecithinase '*C*' of *Clostridium welchii* described by MacFarlane & Knight (1941). This is a phosphodiesterase, releasing phosphocholine, but not free P, from lecithin, and is maximally activated by Ca in concentrations similar to the optimum found for the reaction that splits DPI. Activation by Mg is said to be slight in comparison with the Ca effect.

Inhibition of the release of inorganic P appears to be a non-specific action of bivalent cations, being shown equally by Ca, Mg and Ba, and is presumably related to the increased turbidity of substrate suspensions in media containing these cations. The 5-nucleotidase of brain tissue is also inhibited by Ca and Mg to a similar extent (Pratt, 1954).

Breakdown of potassium diphosphoinositide. The faster reaction with the 'soluble' compound suggests that the rate-limiting factor with the crude substrate is the relative insolubility of the Ca–Mg salt of diphosphoinositide in the medium. The possible relevance of this observation to *in vivo* conditions cannot be judged at present; the Ca and Mg of the crude substrate appear native to it (Folch, 1942, 1949*a*, *b*).

Distribution of enzyme activity. The even distribution of the enzyme system in fairly grossly defined areas of the brain, particularly between white and grey matter, seems to exclude its special participation in highly localized events in the central nervous system. In non-nervous tissues the reaction has not been studied sufficiently to compare it in detail with that occurring in brain preparations. Information is also needed on the distribution and chemical nature of substrates for the enzyme system; inositol-containing lipids have been isolated from several tissues (Taylor & McKibbin, 1953), but their constitution has yet to be investigated.

SUMMARY

1. In a suitable medium the reaction between cerebral-tissue suspensions and the 'inositol phosphatide' fraction of brain kephalin results in the concurrent release of inositol monophosphate and smaller amounts of inorganic phosphate.

2. Inositol monophosphate release is activated by Ca salts, whilst inorganic phosphate release is slightly inhibited by Ca, Mg and Ba salts.

3. The purified potassium salt of diphosphoinositide is attacked by cerebral-tissue suspensions at a rate equivalent to 300 μmoles/g. fresh wt. of tissue/hr., which is at least six times as rapid as the reaction with 'inositol phosphatide'.

4. The enzyme system is present in suspensions prepared from the brains of the guinea pig, rat, rabbit, monkey, sheep and infant and adult humans. No great variations in activity have been found in gross anatomical regions of the central nervous system.

5. Suspensions of liver, spleen and kidney also attack 'inositol phosphatide'.

6. The reaction was not influenced significantly by several enzyme inhibitors, drugs and compounds structurally related to inositol.

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Techniques in Tissue Metabolism

4. APPARATUS FOR MAINTAINING AND RAPIDLY TRANSFERRING TISSUE SECTIONS*

By P. J. Heald and H. McIlwain
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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Enzymes of a given tissue can often cause the labile metabolites in that tissue to react completely in a minute or so. This speed of reaction is especially notable in excitable tissues, and in those of the central nervous system metabolites including lactic acid, phosphocreatine or acetylcholine can be doubled or halved in quantity in 2–10 sec.

Consequently, the study of such substances with preparations of isolated tissues has commonly been carried out under relatively artificial conditions in which the tissue is ground and diluted up to a 1000-fold, and quantities of substrate are added which are very great in comparison with those normally associated with a given quantity of tissue. While these conditions are often excellent for studying individual enzyme reactions, much additional information is usually required before such findings can be applied to the behaviour of the same substances in cell-containing tissues.

An alternative or complementary procedure, with which the present paper is concerned, is to examine events in cell-containing tissues over periods within the few seconds or minutes during which their native metabolites undergo reaction. The general procedure has been to establish a steady state in the tissue, to apply a given agent, and to fix the tissue for analysis at brief intervals afterwards. The agents have been added chemicals or electrical pulses. Such arrangements have in the past been employed with intact organs as muscle or nerve, but rarely with tissue slices.

* Part 3: Rodnight & McIlwain (1954).

EXPERIMENTAL

Tissues and salines

From the guinea pig or larger animals large slices, 0·35 mm. thick, can readily be obtained from the liver, kidney cortex and brain (McIlwain, 1951). Such slices, up to 2 cm. × 1·5 cm. (100 mg.), were strong enough to be picked up on wires and drained of fluid for weighing and transferring. Slices rather larger than the size mentioned were floated from the blade or cutting plate into a shallow dish of the appropriate saline (Rodnight & McIlwain, 1954; Heald, 1954), and trimmed to size with a scalpel, picked out with the rider of a torsion balance and drained on a glass surface, weighed, and returned to the saline. A transfer-holder was opened, its lower jaw put in the saline, the slice was manoeuvred over it with a spatula and lifted from the saline with the holder. The jaw of the holder was then closed on the slice, and the holder and slice were placed into the beaker (Fig. 1) containing about 5 ml. of appropriate saline.

Quick-transfer holders

For rapid transference the tissue was held, before and during transfer, in a holder (Fig. 1) from which it could be easily released. A slice must be kept extended for adequate exchange with its environment, and this was done by fixing it at nine places near each of its long edges. These places were the points of contact between the jaws of the holder (*c* or *d*, Fig. 1) and a nylon thread or metal wire wound round them. The opposing faces of the jaws were double-bevelled so as to form a continuous knife edge *f*, *f'* around each jaw. A cross-section of part of the jaws is shown in Fig. 1*D*. The knife edges closed on the wire or thread wrapped round the opposing jaw. These wires, 4 upper and 5 lower (Fig. 1*C*), formed the nine places along the front and back edges at which the tissue was gripped. The thread or wire also supported the slice, but apart from the points of contact it did so only loosely so that during oxygenation of the medium the slice could be seen to wave gently between the wires. At each point of contact the tissue was distorted as indicated in Fig. 1*D*, but much of this tissue would appear to have been displaced rather than crushed. Provision of several such points minimized tendency to tear at any one of them. In most experiments the holder with its slice fitted into a 30 ml. Pyrex beaker (Fig. 1*B*). Pieces of sponge rubber were cemented permanently to the main strips of the holder so that they formed a seal at the top of the beaker. Through this projected the top of the holder and also a fine plastic tube admitting a gas, usually O_2 or $O_2 + CO_2$ (95:5, v/v), through the fluid in the beaker. The tube was cut at its lower end at an angle to minimize blocking and to direct the stream of bubbles (Fig. 1*A*). When these were provided in an almost continuous stream (50–250 ml. of gas/min.) from the tube in the position shown beside the holder, fluid circulated adequately under and over the slice; the gas escaped round the sponge rubber.

Electrical characteristics. Much use has been made of a type of the holder wound with silver wire, so that electrical potential gradients can be established in tissue held by the apparatus. The type and arrangement of wire followed earlier practice with other electrode types (especially type *H* of Ayres & McIlwain, 1953). Silver wire, 0·315 mm. in diam., was insulated for us by The Temple Electric Co., Watford, Herts, with a plastic enamel. It was threaded through holes and grooves in the holder, as shown in Fig. 1, so that the centres of the wires crossing the jaws were 4 mm. apart. The upper surface of the lower jaw of the holder was crossed by five such wires, and the under surface of the upper jaw by four wires parallel to the others and spaced evenly between them. The opposing surfaces of these parallel sections of the wire were scraped clean of enamel. They thus formed a grid of electrodes, 2 mm. apart, of which alternate members were connected by the remainder of the insulated wire. An end of each coil of the wire was brought to a terminal lug at the top of the holder.

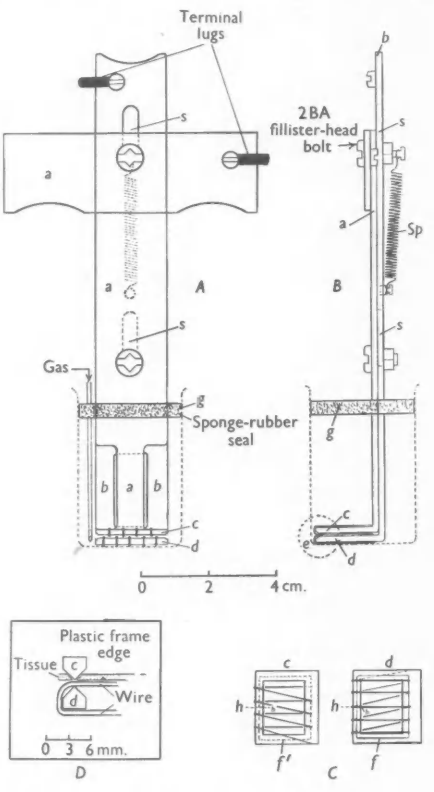

Fig. 1. For legend see bottom of next column.

Fig. 1. Quick-release holder. (*A*) Arrangement of holder and of oxygen tube in beaker (30 ml.). (*B*) End elevation of *A* as viewed from the right-hand side. Perspex strips *a* and *b* (0·0625 in. thick) are held together with two bolts fixed to strip *a*; slots *s* in strip *b* permit the jaws *c*, *d*, to move up to 1·5 cm. Normally the jaws were kept together by the spring *Sp*, and a force of about 250–300 g. was needed to open them. The tops of the plastic strips were shaped as shown (*A*) to take the fingers and thumb of one hand. Just above the jaws, strips *a* and *b* are cut as shown in *A* so as to prevent fluid creeping up between the strips by capillary action. The nuts, bolts, terminals and spring were of 18/8 F. M. B. Staybrite Steel (Firth Vickers). The gas seal *g* was of Rubazote closed-cell sponge rubber (W. Mannering, Bermondsey) cemented to the strips with Araldite D, cold-setting (Aero Research, Duxford). (*C*) Plan of electrode jaws showing arrangement of electrode wires; these were of enamelled-silver wire which was led up the side of the strips *a*, *b*, in milled slots and soldered to the terminal lugs of tinned metal at the top of the holder. Opposing faces of the jaws are double-bevelled to form the knife edges indicated by the lines *f*, *f'*. (*D*) Enlarged view of a section of jaws showing how a slice is held between them. The portion illustrated is that of *c* at the cross-section labelled *hh* in *C*, after assembling the holder and introducing a slice. The compression of the slice occurs only at the places where the wires meet the knife edge of the opposing jaw and involves about 1 sq.mm. of the tissue; the remainder is freely bathed with saline.

Table 1. *Uniformity of metabolic characteristics of tissues in quick-transfer electrodes*

Slices of guinea-pig cerebral cortex of about 120 mg. were in the electrodes in 5 ml. of oxygenated saline at 37·5°. Pulses were alternating (100/sec.), peak potential was 10 v, and time-constant 0·4 msec. Lactic acid was determined according to Barker & Summerson (1941) after preparing the tissue and solution as described by McIlwain & Tresize (1956). Creatine phosphate was determined according to Heald (1954). Values quoted are means ± S.E. with the number of determinations in parentheses. Determinations were made after 60–80 min. incubation; values for lactate of the tissue include that of adhering fluid.

Substance measured	Duration of pulses	Amount (μmoles/g.) No pulses	With pulses
Lactic acid of fluid	60 min.	17·1 ± 0·9 (8)	31·8 ± 1·3 (4)
Lactic acid of tissue	20 sec.	2·60 ± 0·07 (6)	4·65 ± 0·08 (6)
Phosphocreatine of tissue	10 min.	1·45 ± 0·12 (9)	0·75 ± 0·06 (3)

When the quick-transfer electrodes were immersed in saline solutions and potentials applied to them, the potential gradients established were measured as described by Ayres & McIlwain (1953). When condenser impulses or sine-wave alternating currents were applied, potential varied with distance between adjacent wires as recorded in Figs. 5 and 7 of Ayres & McIlwain (1953). In glucose-phosphate saline with sine-wave current at a virtual potential of 2·25 v (6·3 v peak to peak), measurement of the characteristics of eight intervals between adjacent wires gave (± S.D.): sum of abrupt changes between wire and solution, 1·25 ± 0·17 v; gradient over the greater part of the interval, 1·42 ± 0·23 v/mm. (extending for 1·49 ± 0·26 mm.); distance with gradient of 0–1·5 v, 0·3 mm.; highest gradient in the remaining 0·2–0·3 mm., 6·9 ± 1·2 v/mm. Similar uniformity was found between a group of six electrodes, when examined also with condenser pulses of peak potential 10 v, and time-constant 0·4 msec., applied at 100/sec. The electrical characteristics of the electrodes are thus similar to those of electrodes *H* (Ayres & McIlwain, 1953), in which similar wires are arranged at the same distance apart, though in electrodes *H* the wires are shorter and fewer than in the quick-transfer electrodes.

Procedure

Six or eight tissues with holders and beakers concerned in the same experiment were fitted into a frame held in a thermostatically controlled water bath. An experiment was commenced by preparing the saline and distributing it in the beakers, and preparing slices from the chosen organ and fitting them to their holders. Incubation for the desired preliminary period was then commenced and fixing agents were prepared. The appropriate brief treatment (e.g. exposure to pulses for a few seconds) was then applied to one tissue specimen, after which it was immediately fixed by lifting its holder from the beaker, draining it if necessary and then immersing the jaws with their tissue in the fixing agent. At the same time the jaws were opened and a scooping motion was given to holder and beaker of fixing agent so that the tissue floated out from the holder. Other tissue specimens were then treated and fixed.

Metabolic behaviour of tissues in the holder

The following experiments were done to determine (1) the uniformity of behaviour of groups of tissue specimens incubated under these conditions, and (2) whether tissues so handled were comparable in metabolic behaviour to tissues under more conventional conditions. Preliminary experiments (Heald, McIlwain & Nelligan, 1954) showed especially the necessity for vigorous oxygenation and the provision of the sponge-rubber seal to maintain an atmosphere of oxygen above the tissue. Table 1 lists determinations of lactic acid in the tissues and in the medium, and of creatine phosphate in the tissue both in the presence and

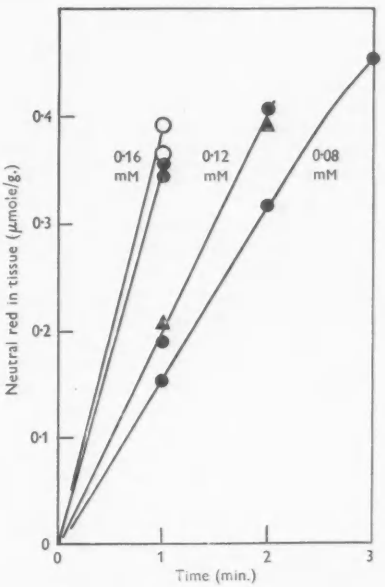

Fig. 2. Uptake of neutral red by guinea-pig cerebral cortex. Slices of cortex of about 120 mg. were held in quick-transfer electrodes in 5 ml. of phosphate saline 10 mM in glucose, except when stated otherwise. After 20 min. at 37·5° (zero time), neutral red in the same saline 0·2 ml. was added to give the concentrations stated. Slices were removed at intervals, briefly rinsed, and the dye was extracted and determined according to McIlwain & Grinyer (1950). ●, Glucose and no pulses; ▲, glucose and pulses as in Table 1; ○, no glucose and no pulses.

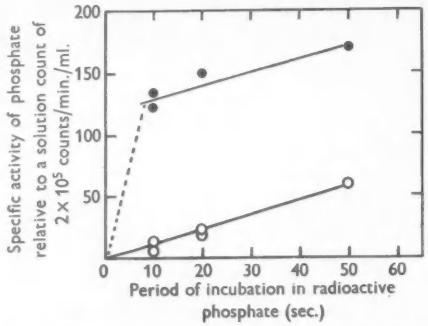

Fig. 3. The incoporation of radioactive phosphate into adenosine triphosphate (γ-P) (○) and tissue orthophosphate (●) of liver slices from fasted rats. Slices were incubated in a glucose-containing saline buffered with aminotrishydroxymethylmethane at 37·5° for 30 min. in O_2 before addition of radioactive phosphate. For procedure and analytical methods see Heald (1956*a*).

absence of electrical pulses. In the absence of pulses, creatine phosphate was maintained at levels found previously with more conventional manometric apparatus where oxygenation may be presumed to be completely adequate (McIlwain, Buchel & Cheshire, 1951; McIlwain & Gore, 1951). Levels of lactic acid in solution in absence of pulses were somwhat lower than those found previously in manometric apparatus (McIlwain, Anguiano & Cheshire, 1951). Passage of pulses through the slices doubled their lactic acid and halved their creatine phosphate content. These changes have been shown to occur in cerebral tissues in manometric apparatus (McIlwain, Anguiano & Cheshire, 1951; Heald, 1953; McIlwain & Gore, 1951). Variation between results in different holders is seen to be small. In addition, incorporation of radioative phosphate into liver slices and of neutral red into cerebral slices was studied, as shown in Figs. 2 and 3. These processes, initiated by additions to the saline surrounding the tissues, proved to proceed uniformly with time. Other investigations employing the holder have been reported by Heald (1954, 1956*b*); Greengard & McIlwain (1955); LeBaron (1955) and McIlwain & Tresize (1956).

DISCUSSION

In most biochemical work with sliced tissues, these have been floating freely in saline solutions, even when relatively brief exposure to added agents was desired. Mechanical restraint of tissues for special purposes has however been described: thus in applying electrical pulses (McIlwain, 1951) or working with non-aqueous fluids (Rodnight & McIlwain, 1954) tissues have been held mechanically without thereby interfering with their normal metabolic activities. The results of the present paper show that the mechanical arrangements and restraint for rapidly transferring the tissue are equally innocuous.

The special virtue of these arrangements lies in the facilitation of the study of changes occurring in periods one-hundredth or one-thousandth of those used in most metabolic investigations. Studies with such a time-scale, e.g. 1 sec. to 1 min., are of especial importance in excitable tissues, and are facilitated in such tissues by their being excitable: for this enables a change of metabolic state to be induced by a non-chemical agent, without problems of diffusion to the tissue, and in a time measured in msec. However, in both excitable and non-excitable tissues the present findings show that added substances do in fact reach the tissue cells from a surrounding fluid very quickly. Thus radioactive inorganic phosphate appeared in the adenosine triphosphate of liver slices 10 sec. after its addition to a fluid which bathed them.

In higher animals the body as a whole survives only for a minute or so without material exchange with its environment, and can respond to administered chemicals in a few seconds; most tissues respire at rates sufficient to exhaust their native oxygen in perhaps 10 sec. There is thus much scope for study of animal tissues within periods of this brevity.

SUMMARY

1. A tissue-holding apparatus is described by means of which thin slices of tissue can, after incubation in a suitable saline, be rapidly transferred to a fixing agent or to another saline.

2. While in the holder the tissue may be subjected to brief exposure, of from 1 sec. upwards, to other agents such as radioactive phosphate or electrical pulses, and can then be removed and fixed within 0·2–0·3 sec. of such exposure.

3. With cerebral tissues it has been shown that the normal levels of creatine phosphate and the process of glycolysis are not affected by the holder.

4. The use of the holder in following the accumulation of neutral red by cerebral slices, and in the incorporation of radioactive phosphate into liver adenosine triphosphate within 10 sec. is described.

We are greatly indebted to Mr D. Nelligan for his taking part in the construction and design of the quick-transfer holder.

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Analysis of Radioactive Phosphates in Extracts of Cerebral Tissues

By P. J. HEALD

Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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As a means of studying the metabolism of radioactive phosphates in extracts of cerebral tissue, a method of analysis was required applicable to the fractionation of small quantities (120–130 μg. of total P), and specific for adenosine triphosphate, inorganic orthophosphate and creatine phosphate; it was also desirable that it should be capable of easy extension to a survey of other compounds or groups of compounds.

The methods in general use for the analysis of phosphorylated intermediates, though of considerable value, suffer not only from the relatively large amounts of tissue necessary but also from the inherent defect of overlapping of fractions (Greenberg, 1952). This, though small, becomes important when studies of the distribution of radioactive phosphates is to be considered (Abood & Gerard, 1952; Ennor & Rosenberg, 1952; Lee & Eiler, 1953; Sacks, 1949). To overcome these difficulties it was decided to try preliminary fractionation of phosphates into groups by means of barium followed by paper chromatography.

The results of Turba & Enenkel (1951), Maurer & Schild (1951), and Schild & Bottenbruch (1953) suggested that ionophoresis on paper would achieve a rapid and complete separation of the phosphates, and studies were commenced along these lines. Recent work (Neil & Walker, 1954; Wade & Morgan, 1954, 1955), has shown the separation of phosphates by paper ionophoresis, but the application to the fractionation of tissue extracts has not been reported.

MATERIALS AND METHODS

Adenosine triphosphate (*ATP*). Three samples of ATP were used. Preparation 1 was prepared from the dibarium salt supplied by Boots Pure Drug Co., by means of the ion-exchange resin Deacidite FF (Permutit Co. Ltd.) with the HCl–NaCl eluants recommended by Cohn & Carter (1950). ATP was precipitated as the barium salt from the fraction removed by 0·2 M-NaCl–0·01 N-HCl and after conversion into the potassium salt with potassium sulphate was stored in solution at $-20°$. The solution showed a sharp symmetrical absorption curve with a peak at 260 mμ. The acid-labile phosphorus determined by 10 min. hydrolysis in N-H_2SO_4 was 68% of the total phosphorus. A preparation of rabbit-muscle myosin liberated 43% of the labile P in 1 hr. and 43·5% in 1·5 hr. When subjected to paper ionophoresis at pH 4·3 the solution showed only a single phosphate-containing compound. Inorganic orthophosphate was absent. The product was considered to be pure ATP.

Preparation 2 was prepared from rabbit muscle by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). The final product contained 64% acid-labile phosphorus, and it migrated mainly as a single spot on ionophoresis at pH 4·3. A faint spot corresponding to adenosine diphosphate (ADP) was present. Inorganic orthophosphate was absent.

Preparation 3 was a chromatographically pure sample of the crystalline sodium salt, and was a gift from Sigma Chemical Co., St Louis, U.S.A.

Adenosine diphosphate (ADP) was also a gift from Sigma Chemical Co. of the chromatographically pure sodium salt.

Creatine phosphate was prepared from creatine by the method of Ziele & Fawaz (1938). The product obtained as the calcium salt was stored over P_2O_5.

Glucose 1-phosphate was a gift of the pure dipotassium salt from Roche Products Ltd. The material migrated as a single spot on ionophoresis at pH 4·0 and pH 8·6, and contained 99·9% of the theoretical phosphorus content. Inorganic orthophosphate was absent.

Glucose 6-phosphate and fructose 6-phosphate were obtained as a mixed sample of the barium salts from Dr C. Long.

Myosin and potato apyrase. Myosin was prepared from rabbit muscle by the method of Bailey (1942). However, in view of the laboriousness of the preparation and the rapid loss of activity on storage the use of myosin was

discontinued. Instead, a preparation of potato apyrase was made and tested by the method of Lee & Eiler (1951). The results obtained with this preparation were in good agreement with those of Lee & Eiler (1951, 1953).

Determination of phosphorus. Orthophosphate was determined by the method of Berenblum & Chain (1938) as modified by Long (1943). Total phosphorus was determined by digestion of the samples (0·5 ml.) with 0·5 ml. of the sulphuric–perchloric acid digestion mixture of Hanes & Isherwood (1949). After digestion the cooled solutions were adjusted to the phenolphthalein end-point with 20% (w/v) NaOH and diluted to 5–6 ml. with water. 10N-H_2SO_4 was then added to adjust the final acid concentration to N. The solutions were heated for 20–30 min. at 100° and cooled, and orthophosphate was estimated as above. To estimate total P in areas of paper after ionophoresis, the paper was first ashed (Wade & Morgan, 1954) before addition of the digestion mixture. By carrying out the whole procedure in Pyrex tubes (110 mm. × 20 mm.) the estimation of P was accomplished without transfer of solutions.

Paper ionophoresis was carried out either on Whatman no. 1 or no. 3MM paper. The latter paper was used for quantitative work. Before use all papers were washed first with N-HCl followed by 0·5% ethylenediaminetetraacetic acid (versene) in N-NH_3 soln., N-HCl, N acetic acid, and finally with glass-distilled water. A wash with glass-distilled water was given between each acid or alkaline wash. This treatment reduced the phosphorus content of the paper to 0·02–0·03 μg. of P/sq.cm.

The buffers used were either 0·05M Na acetate–acetic acid (pH 4·0), or 0·05M aminotrishydroxymethylmethane (tris) adjusted to pH 8·6 with HCl. The applied potential was 350–450 V and a current density of 0·5–0·8 mA/cm. was maintained for 6–7 hr. The papers were removed, dried at 80° and developed either as described by Bandurski & Axelrod (1951) or by spraying with 0·25% (w/v) ammonium molybdate in N-H_2SO_4, and reheating at 80° until dry, respraying lightly and finally developing under an ultraviolet lamp (Osram or Mazda), from which the Woods glass filter had been removed. The phosphates appeared blue on a white background. With quantitative chromatograms from which the phosphates were to be eluted, guide strips of the pure phosphates were included in the run. These were later cut off and developed separately.

Extraction of phosphates from cerebral tissue and fractionation of the extract

Cerebral tissues (200–300 mg. wet wt.) were transferred to 3 ml. of ice-cold 10% (w/v) trichloroacetic acid (TCA) and were brought into a fine suspension by grinding in a glass tube with a fitting pestle. The suspension was centrifuged at 25 000 *g*, at −5° in the superspeed head of the M.S.E. Major refrigerated centrifuge, for 15 min. The residues were re-extracted with 2 ml. of 5% (w/v) TCA and the combined supernatants were adjusted to a definite pink phenolphthalein end-point with 5N and 0·1N-KOH. Then 1 ml. of 25% (w/v) barium acetate was added; the suspension was kept at 0° for 15 min. and was then centrifuged at 4500 *g* at 0° for 15 min. The residue was dissolved in 2 ml. of 0·1N-HCl and reprecipitated at pH 8·0 with 0·1 ml. of barium acetate solution. After 15 min. at 0° the residue was centrifuged and yielded the phosphates with insoluble barium salts.

The combined supernatants were precipitated with 5 vol. of 95% (w/v) ethanol, and sufficient 5N-KOH was added to produce a definite pink end-point to phenolphthalein. The solution, after 1–2 hr. at 0°, was centrifuged, yielding the phosphates with barium salts soluble in water but insoluble in ethanol.

Removal of barium from the residue

Phosphates with barium salts insoluble in water. This fraction was suspended in 3 ml. of water and shaken with 200–300 mg. of Zeo-Karb 315 (H^+) (90–120 mesh) (Permutit Co. Ltd., London) for 3–5 min. at 2°. The suspension was centrifuged and the residue washed twice with 2 ml. of water. The combined supernatants were transferred to a glass bulb tapering to a point, and a drop of conc. NH_3 soln. was added and the solution frozen at −20°. The water was evaporated under vacuum over P_2O_5 at 2°. The residue was taken up in 0·1 or 0·2 ml. of water, centrifuged to remove some impurities and the supernatants were measured on to paper.

Phosphates with barium salts soluble in water but precipitated by ethanol. This fraction was dissolved in 3 ml. of water at 0° and shaken with 200–300 mg. of Zeo-Karb 315 (NH_4^+) for 1 min. It was centrifuged promptly at 0°, the residue extracted twice with 2 ml. of ice-cold water and the supernatants were treated as described above. In view of the lability of creatine phosphate this operation was performed as rapidly as possible.

Tissues and salines

Slices of cerebral cortex of guinea pigs were cut as described previously by McIlwain (1951) and were incubated in conical flasks in a saline buffered with tris (Heald, 1954) for 30 min. For experiments in which concentrations of phosphates *in vivo* were to be determined, guinea pigs (100–200 g. wt.) were drowned in liquid oxygen and samples of whole brain were obtained as described by Kratzing & Narayanaswami (1953).

RESULTS

Ionophoresis of phosphates. In Table 1 are shown the R_P values of phosphates, most of which have been found to occur in extracts of brain (Stone, 1943; LePage, 1946), when subjected to ionophoresis at two different pH values. The figures quoted do not give an indication of the degree of overlap of two components moving close together. Thus ATP and inorganic phosphate (P_i) though apparently separated in acetate at pH 4·0 nevertheless suffered from a degree of overlap (cf. Wade & Morgan, 1954). At pH 8·6 ATP and ADP were not separable but were consistently separated from P_i. At this pH also, P_i, creatine phosphate, adenylic acid, diphosphopyridine nucleotide (DPN) and the hexose monophosphates were well separated. The hexose monophosphates were not separable from one another at this pH (cf. Neil & Walker, 1954). In experiments, not shown, at pH 7·4 in tris buffer, the following groups of phosphates were not separated: ATP, ADP and P_i; creatine phosphate and hexose monophosphates.

Precipitation of the phosphates with barium salts insoluble in water. In Table 2 are shown the amounts of P_i precipitated by 1 ml. of 25% (w/v) barium acetate in 15 min. at 0° from trichloroacetic acid solutions made alkaline with KOH. The volumes did not exceed 10 ml. The amounts of P_i and ATP used are such as are likely to occur in work with cerebral slices. In all experiments, the residues from the first precipitation were redissolved in 2·0 ml. of ice-cold 0·1 N-HCl and reprecipitated by adding 0·1 ml. of 25% (w/v) barium acetate and making alkaline with KOH (Cori & Cori, 1931).

Although the recoveries appeared adequate (92–94%), experiments with $^{32}P_i$ present showed that appreciable amounts were not precipitated but remained in the supernatants; increasing the amount of carrier P_i fivefold reduced the amount of $^{32}P_i$ escaping precipitation only by half. The difference between the two types of estimation is attributed to the failure of the method of phosphate estimation to detect differences of the order of 0·2–0·5 μg.

Table 1. *Rate of movement of phosphates relative to the rate of movement of inorganic orthophosphate (R_P) during paper ionophoresis*

Applied potential: 400–450 v. Current density: 0·5–0·8 mA/cm. Buffers, 0·05 M. Paper, Whatman no. 3MM. Average length of run, 6 hr. Values are calculated from the relative distances of the centres of the phosphate spots.

Phosphate	R_P value: In acetate at pH 4·0	R_P value: In tris buffer at pH 8·6
Orthophosphate	1·0	1·0
Adenosine triphosphate	0·90	0·70
Adenosine diphosphate	0·75	0·70
α-Glycerophosphate	—	1·0
Fructose 1:6-diphosphate	1·0	1·0
Creatine phosphate	—	0·88
Glucose 6-phosphate / Fructose 6-phosphate	0·72	0·75
Adenylic acid	—	0·61
Diphosphopyridine nucleotide	—	0·31
Ethanolamine phosphate	0·61	0·67
Inosine triphosphate	0·90	0·70

Precipitation of ATP under similar conditions, at a quantity of 1·0 μmole/ml., resulted in 1·57 ± 0·2% (4) of the amount taken remaining in the supernatant. This quantity was considered to be sufficiently small to be neglected. Ennor & Stocken (1948) similarly found that the precipitation of ATP by barium was virtually quantitative. KOH was used for neutralization of the trichloroacetic acid solution since the use of NaOH with muscle extracts results in incomplete precipitation of P_i at pH 8·2 (Kosterlitz & Ritchie, 1943; Ennor & Stocken, 1948).

Precipitation of ATP and P_i together was found to be almost quantitative; thus with 0·55 μmole of P_i and 0·8 μmole of ATP added, recoveries were 0·556 ± 0·007 μmole of P_i and 0·82 ± 0·022 (5) μmole of ATP; and with 0·487 μmole of P_i and 0·354 μmole of ATP added, recoveries were 0·467 ± 0·029 (6) μmole of P_i and 0·330 ± 0·017 (6) μmoles of ATP.

Precipitation of the phosphates with barium salts soluble in water but precipitated by ethanol. The phosphates used to study the precipitation of this fraction were pure glucose 1-phosphate and creatine phosphate. In a final volume of 10–12 ml. there was little difference in the amounts of glucose 1-phosphate precipitated by 4 or 5 vol. of alkaline ethanol, but a marked difference when the final vol. was 50–60 ml. (Table 3).

The precipitation of creatine phosphate from solution was incomplete in the absence of a carrier phosphate, but was quantitative in the presence of glucose 1-phosphate. Thus, in a final vol. of 60 ml., the precipitation of barium creatine phosphate by 5 vol. of alkaline ethanol yielded these results: with carrier absent and 0·362 μmole of creatine phosphate present, recoveries were 0·168 μmole with a range of 0·115–0·213 μmole: with 0·815 μmole of glucose 1-phosphate present, recoveries were 0·393 ± 0·045 (5) μmole of creatine phosphate.

Removal of barium from the precipitates. The presence of traces of insoluble barium salts is known to cause marked streaking of chromatograms of phosphate esters (Hanes & Isherwood, 1949), and this occurred with paper ionophoresis at

Table 2. *Recovery of inorganic and radioactive orthophosphate when precipitated as the barium salts from 10 ml. of solution*

Conditions as described in Methods. Values are given ± S.E.M. Figures in parentheses indicate the number of determinations.

P_i added (μmole): With $^{32}P_i$	P_i added (μmole): Without $^{32}P_i$	P_i found in residue (μmole)	$^{32}P_i$ found (counts/min. as % of counts/min. taken): In residue	$^{32}P_i$ found (counts/min. as % of counts/min. taken): In supernatant
—	0·20	0·21 ± 0·003 (7)	—	—
—	0·23	0·21 ± 0·004 (4)	—	—
—	1·03	0·95 ± 0·004 (4)	—	—
0·49	—	—	88·2 ± 0·3 (8)	11·8 ± 0·5 (8)
2·65	—	—	94·6 ± 0·7 (6)	5·3 ± 0·7 (6)

pH 8·6. Exceptions to this were the calcium and barium salts of creatine phosphate and of glucose 1-phosphate which, being soluble in water, permit a movement of the cation towards the negative electrode while the phosphate moves towards the positive electrode. However, excess barium precipitated, presumably as carbonate, with the phosphates necessitated removal before ionophoresis. It was found simplest to remove barium by decomposition with a cation-exchange resin.

The recovery of ATP, P_i and creatine phosphate after precipitation as the barium salt and decomposition with the resin Zeo-Karb 315 is shown in Table 4. It will be seen that recoveries of ATP in the presence and absence of P_i was some 76%, while the recovery of P_i was 85–90%. Recoveries of 82% were obtained for creatine phosphate, some of which had decomposed during the treatment. In early experiments it was found that treatment of pure ATP (Preparation 3) for 30–40 min. at 0° with Zeo-Karb 315 led to the formation of a compound which moved slightly faster than ATP. This compound showed a sharp absorption band with a peak at 260 mμ. and had a ratio of acid labile P to total P of 7:10 (cf. Lund, Grylls & Harrison, 1954). The quantity of the material was reduced if the period of contact with the resin was reduced.

The solutions after treatment could be freeze-dried to yield residues which when taken up in water and subjected to ionophoresis showed well-defined spots without any streaking. The recoveries of ATP and of creatine phosphate when solutions were subjected to the whole procedure are about 80% (Table 5). This was considered satisfactory in view of the number of steps involved.

Table 3. *Recovery of glucose* 1*-phosphate by precipitation of the barium salt from alkaline ethanolic solution at* 0°

Values are given ± S.E.M. Figures in parentheses indicate the number of experiments.

Glucose 1-phosphate added (μmole)	Ethanol added to aq. soln. (vol.)	Final concn. of ethanol soln. (%)	Final vol. of solution (ml.)	Glucose 1-phosphate	
				Found (μmole)	Recovered (%)
0·25	4·0	76	10	0·19 ± 0·004 (3)	76
0·25	5·0	80	12	0·20 ± 0·004 (9)	80
0·49	4·0	76	10	0·42 ± 0·009 (3)	86
0·49	5·0	80	12	0·45 ± 0·013 (7)	92
0·49	4·0	76	50	0·35 ± 0·035 (6)	71
0·49	5·0	80	60	0·40 ± 0·027 (7)	82

Table 4. *Recovery of adenosine triphosphate, orthophosphate and of creatine phosphate after precipitation of the barium salts and decomposition with Zeo-Karb* 315

The ATP and P_i precipitate was decomposed with Zeo-Karb 315 (hydrogen-ion form), while the creatine phosphate (CrP) precipitate was decomposed with Zeo-Karb 315 (ammonium form). The latter phosphate was precipitated in the presence of 1·29 μmoles of hexose monophosphate. Values are given ± S.E.M. Figures in parentheses are the number of determinations.

Phosphate added (μmole)			Phosphate recovered (μmole)		
P_i	ATP	CrP	P_i	ATP	CrP
0	0·31	0	0	0·24 ± 0·016 (4)	0
0·48	0·31	0	0·44 ± 0·009 (5)	0·24 ± 0·014 (5)	0
0·48	0·63	0	0·42 ± 0·006 (3)	0·45 ± 0·01 (3)	0
0	0	0·63	0·029	0	0·52 ± 0·011 (6)

Table 5. *Recovery of adenosine triphosphate and creatine phosphate after precipitation as the barium salts, and decomposition with Zeo-Karb* 315*, and paper ionophoresis at* pH 8·6

Phosphate was determined as total phosphate after location on the paper strips. Values are given ± S.E.M. Figures in parentheses indicate the number of determinations.

Phosphate taken (μmole)		Phosphate found (μmole)		Phosphate found (as % of amount taken)	
ATP	CrP	ATP	CrP	ATP	CrP
0·52	0	0·44 ± 0·014 (5)	0	84 ± 2·7 (5)	0
0·78	0	0·61 ± 0·03 (6)	0	79 ± 4·1 (6)	0
0	0·63	0	0·52 ± 0·03 (6)	0	83 ± 5·5 (6)

Analysis of cerebral tissue

Tissues were denatured in ice-cold trichloroacetic acid, and were then centrifuged to remove debris. The efficiency of this process in two different centrifugal fields is shown in Table 6. In a high centrifugal field, removal of debris was complete and the solutions were quite clear. Very little additional phosphorus was extracted from the residue by a further two or three extractions. In contrast in a low centrifugal field, the supernatants are opaque and a second extraction yielded 50% as much phosphorus as the first extraction. This material was phospholipid (G. H. Sloane-Stanley, personal communication). It seems clear that with trichloroacetic acid extracts of cerebral tissues, the use of low centrifugal fields can lead to spuriously high values for the extractable phosphorus.

Qualitative examination of phosphates found when extracts were subjected to the above procedure showed spots corresponding to ATP and ADP, P_i, hexose monophosphate, creatine phosphate and a group labelled '*A*' containing both P_i which was not entirely precipitated by barium and probably any triose phosphates present. The phosphates soluble in ethanol were not examined further beyond ascertaining the quantity of phosphorus present. The area corresponding to ATP could have contained inosine triphosphate, since this was not separable from ATP. However, an eluate of the area showed a single sharp absorption peak at 260 mμ., the quotient E_{275}/E_{260} being 0·46, which compares with a value of 0·41 reported by Schmitz, Hurlbert & Potter (1954). In addition, fractionation of the phosphates having insoluble barium salts on a Dowex-1 column by the method of Utter, Kurahashi & Rose (1954) failed to reveal any material absorbing at 250 mμ. in eluates which showed such absorption when on authentic sample of inosine triphosphate was fractionated on the same column. Inosine triphosphate was considered to be absent.

The identity of the phosphate in the area corresponding to creatine phosphate was established by elution and subsequent determination of the creatine/phosphorus ratio. In two experiments this ratio was 1·0 and 0·95 respectively, from which it was concluded that the phosphate was creatine phosphate. No attempt was made to elute and identify the hexose monophosphate group.

Quantitative analysis of phosphates. The results of the separation and estimation of phosphate in cerebral tissue from guinea pigs are shown in Table 7 and are compared with similar data found for guinea-pig brain by other authors who had used different techniques. The values found are generally in good agreement with values obtained by other workers. A noticeable exception is the hexose monophosphate group, which will be discussed below. The group '*A*' forms too large a quantity to consist merely of P_i that escapes precipitation by barium, and probably contains triose phosphates.

The phosphate added to the paper was accounted for in the fractions estimated. Thus for the phosphates with insoluble barium salts $103 \pm 3{\cdot}8$% (4) of the phosphate added to the paper was accounted for as the sum of ATP and P_i, and for the phosphates soluble in water but precipitated by ethanol $96{\cdot}1 \pm 7{\cdot}5$% (6) was accounted for. Later experiments with $^{32}P_i$ confirmed these results and showed that no labelled phosphorylated intermediates could be detected as being absorbed by the resins (Heald, 1956).

In Table 8 is shown the degree of contamination of ATP, creatine phosphate and the hexose monophosphates with $^{32}P_i$ which was added to trichloroacetic acid extracts of cerebral tissue before fractionation. It is seen that the amount of contaminating ^{32}P appearing in the above compounds was well below that necessary to make the method of value in studies on the incorporation of ^{32}P into these fractions. Further, the results show that the use of an ion-exchange resin to remove barium did not promote any exchange between the ^{32}P in solution and phosphorus of the fractions. Not all the ^{32}P added was precipitated even by ethanol, for $1{\cdot}3 \pm 0{\cdot}3$% (4) of a count of 25 000 counts/min. added to a trichloroacetic acid extract was recovered in the fraction containing phosphates whose barium salts are soluble in water and ethanol. Whether this implies that inorganic phosphate is incompletely precipitated by ethanol and barium, or whether the samples of ^{32}P obtained contained other substances, is not known.

Table 6. *Effect of different centrifugal fields on the apparent amount of acid-soluble phosphorus extracted from cerebral tissue*

Slices of cerebral cortex were denatured in 10% (w/v) trichloroacetic acid, and the residues re-extracted with trichloroacetic acid. Total phosphorus was determined in each extract.

	P in extract (μmoles/g. wet wt. of tissue)			
	3500 *g*		25 000 *g*	
Extraction	(1)	(2)	(1)	(2)
1st	9·1	7·9	12·3	11·2
2nd	4·6	3·2	0·44	0·62
3rd	3·1	2·4	0·34	0·19
4th	2·3	2·7	—	—
5th	1·4	1·7	—	—
Total phosphorus in all extracts	20·5	17·9	13·08	12·01

Table 7. *Phosphates of guinea-pig brain* in vivo *and* in vitro

For details of analysis see Methods. Values marked with an asterisk were obtained from tissues respiring in a phosphate-containing saline. Values are quoted ± S.E.M. Figures in parentheses indicate the number of determinations. Fraction '*A*' is given in terms of μmole of P/g. wet wt. of tissue.

Tissue preparation	Phosphates (μmoles/g. wet wt. of tissue)							Method of estimation	Authors
	ATP and ADP	P_i	CrP	Hexose monophosphates	Adenylic acid	DPN	'*A*'		
Cerebral-cortex slices *in vitro*	0·80 ±0·03 (7)	2·15 ±0·21 (10)	1·12 ±0·14 (9)	0·72 ±0·10 (9)	0·59 ±0·11 (7)	0·17 ±0·04 (5)	0·91 ±0·09 (6)	Ionophoresis on paper	Present work
	—	1·52 ±0·14 (14)	1·35 ±0·08 (14)	—	—	—	—	Barium separation	Heald (1954)
	—	3·08* ±0·31 (15)	1·46 ±0·2 (15)	—	—	—	—	Calcium ethanol separation	McIlwain, Buchel & Cheshire (1951)
	1·01 ±0·07 (5)	—	1·12 ±0·04 (6)	0·23	—	—	—	Enzymic	Kratzing & Narayanaswami (1953)
	1·09 ±0·09 (6)	4·92* ±0·39 (6)	1·19 ±0·09 (4)	—	—	—	—	Calcium ethanol separation	Kratzing & Narayanaswami (1953)
Cerebral-cortex slices									
(*a*) Fresh slices	—	—	—	—	—	0·23	—	Manometric	Gore, Ibbott & McIlwain (1950)
(*b*) In glucose saline	—	—	—	—	—	0·20	—	Manometric	Gore, Ibbott & McIlwain (1950)
Whole brain frozen *in vivo*	3·5 ±0·1 (3)	3·2 ±0·4 (3)	2·68 ±0·12 (4)	1·34 ±0·21 (4)	1·02 ±0·08 (4)	—	—	Ionophoresis on paper	Present work
	—	3·8	3·3	—	—	—	—	Calcium ethanol separation	McIlwain *et al.* (1951)
	3·28	—	2·14	0·26	—	—	—	Enzymic	Kratzing & Narayanaswami (1953)
	3·68	3·58	2·66	—	—	—	—	Calcium ethanol separation	Kratzing & Narayanaswami (1953)

Table 8. *Degree of contamination of adenosine triphosphate, creatine phosphate and hexose monophosphate in tissue extracts when separated from radioactive orthophosphate by precipitation and ionophoresis on paper*

For details see Methods. CrP: creatine phosphate; HMP: hexose monophosphate.

Determination	Counts/min. in area estimated				Counts/min. as % of $^{32}P_i$ added		
	ATP	CrP	HMP	P_i	ATP	CrP	HMP
1	11·0	—	—	9 136	0·12	—	—
2	23·5	28·0	7·6	10 297	0·22	0·27	0·07
3	5·7	0·0	3·0	6 000	0·09	0·0	0·05

DISCUSSION

The method of analysis of phosphates described here, although based on an initial separation of the phosphates into groups by means of barium fractionation, overcomes most of the defects inherent in that procedure when applied to extracts containing radioactive phosphate in various forms. Thus the further separation of the fractions by means of paper ionophoresis yields compounds or groups of compounds which can either be estimated after acid digestion or be eluted and analysed by other methods.

Separation of phosphates other than creatine phosphate into groups by barium fractionation and subsequent paper-partition chromatography has been employed by Bandurski & Axelrod (1951) and by Ganguli (1953). Ionophoresis on paper at pH 3·2 has been used by Wade & Morgan (1955), though at this pH creatine phosphate is slowly dephosphorylated, resulting in 'streaking' between this ester and orthophosphate. A similar result attends the use of the Hanes & Isherwood (1949) solvents (Caldwell, 1953). In this respect ionophoresis at pH 8·6 appears to be advantageous.

Separation of the groups of phosphates containing adenosine di- and tri-phosphates and orthophosphate and hexose diphosphate is not complete, the resolution being into the adenyl phosphates, and orthophosphate plus hexose diphosphate. However, the use of an enzymic method for the determination of the terminal phosphate of adenosine triphosphate reduces possible errors of contamination by adenosine diphosphate. In cerebral extracts hexose diphosphate is reported as being absent or present in extremely low quantity, i.e. 0·1 μmole/g. wet wt. (LePage, 1946; Albaum, Tepperman & Bodansky, 1946), and does not constitute a grave error if included with orthophosphate.

The quantities of hexose phosphates found by the methods described above are markedly greater than those found by Kratzing & Narayanaswami (1953), whose value included any triose phosphates present in the extracts. Since their method of analysis must be presumed to estimate all the hexose monophosphate present (cf. Slater, 1953) the only obvious difference lies in the preparation of the extract. Thus Kratzing & Narayanaswami denatured the tissue in ice-cold perchloric acid and removed the perchlorate by conversion into the potassium salt and cooling in ice, when a large part of the potassium perchlorate crystallized. Possibly perchloric acid does not extract all the phosphates soluble in trichloroacetic acid, or the crystalline perchlorate adsorbs some phosphates such as hexose phosphates.

SUMMARY

1. A system of analysis of acid-soluble phosphates found in cerebral tissues, based on a paper-ionophoretic method, is described for use in the study of the metabolism of radioactive phosphorus.

2. Trichloroacetic acid extracts of tissue are first fractionated into groups by barium precipitation. The barium salts are then decomposed by a cation-exchange resin, and the resulting solutions are freeze-dried, before separation of the phosphates by ionophoresis.

3. The analysis of guinea-pig cerebral tissue by the method yielded results agreeing with values determined by other methods for some of the phosphates.

4. The separation and precipitation of the barium salts of several phosphates has been re-examined from the point of view of the small quantities of the phosphates normally encountered in the analysis of cerebral slices.

5. The centrifuging procedure employed can have a marked effect on the apparent total acid-soluble phosphorus extractable by trichloroacetic acid.

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Effects of Electrical Pulses on the Distribution of Radioactive Phosphate in Cerebral Tissues

By P. J. HEALD
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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It is now well established that in nervous tissue, as in muscle, the application of fluctuating electrical potentials results in marked changes in the levels, both of creatine phosphate and of tissue inorganic phosphate (see McIlwain, 1952, for review). Thus in both tissues the level of creatine phosphate falls while that of inorganic phosphate rises. Information regarding the nature of this change in cerebral tissues has, however, been lacking, and the questions of whether the increased inorganic phosphate does indeed arise from creatine phosphate, and of the route through which the phosphate of creatine phosphate passes during breakdown, do not appear to have been investigated. The problem assumes a greater significance when it is realized that the tissues containing creatine phosphate are those which, owing to the suddenness of events taking place, require a large and immediate source of energy.

In muscle the energy used appears largely in the form of mechanical work, but in cerebral tissues the energy requirements appear to be for the maintenance or re-establishment of a steady state in the continuous ionic changes associated with the manifestation of electrical activity detectable, for example, by the electroencephalograph. Initially, therefore, it might not seem unreasonable to suppose that the pathway of creatine phosphate metabolism in brain may differ in some respects from that in muscle.

During an investigation of the rapid changes in slices of cerebral cortex of guinea pigs brought about by electrical pulses (Heald, 1954) it was found that the rate of breakdown of creatine phosphate appeared to be higher than that required to provide the 'energy-rich' phosphate involved in the metabolism of glucose. From this it was suggested that the 'energy-rich' phosphate released in response to pulses was not used solely in the phosphorylation of glucose. In an attempt to study this problem further, use has been made of radioactive phosphorus and the results obtained are described in this paper.

MATERIALS AND METHODS

Adenosine triphosphate, creatine phosphate and glucose 1-phosphate. These were samples described previously (Heald, 1956).

Radioactive orthophosphate. This was obtained as carrier-free orthophosphoric acid in dilute HCl, from the Radiochemical Centre, Amersham, Bucks. It was diluted to a convenient volume and stored in the refrigerator. Immediately before use a sample was heated with N-HCl for 20 min. at 100° to convert any pyrophosphate into orthophosphate, and cooled, and neutralized with an equal quantity of N-NaOH (cf. Ennor & Rosenberg. 1954). It was diluted with water to a concentration of 10 μc/ml. Samples of such solutions when tested by paper ionophoresis with sodium orthophosphate as carrier yielded no area of activity other than the orthophosphate area.

Tissues and salines. Guinea-pig cerebral slices were cut as described by McIlwain (1951) and were incubated in glucose salines buffered with aminotrishydroxymethylmethane (Heald, 1954). Usually the medium containing no added phosphate was used.

Technique of manipulation. Slices were floated into tissue holders placed in a beaker containing the saline and incubated in a thermostat (Heald, McIlwain & Nelligan, 1954). After 30 min. at 37·5° in O_2, 0·2 ml. of a carrier-free solution of radioactive phosphate was added rapidly from a tuberculin syringe, and incubation was continued for the required period. The amount of NaCl added with the radioactive phosphate did not produce a change in concentration in the medium of more than 3%, and frequently the quantity was sufficient to produce a change of only 1%. The amount of phosphorus added was up to 0·2 μg. After a suitable period of incubation the slices were either transferred to 3 ml. of ice-cold 10% (w/v) trichloroacetic acid or else were rapidly transferred to 20 ml. of fresh saline contained in a small constant-temperature bath at 37·5° and stirred by a stream of O_2. After 10 sec. incubation the holder and slice were transferred to a fresh 5 ml. of saline in a second beaker and re-incubated under O_2 for different periods as required. Slices were then either removed without further treatment and fixed in trichloroacetic acid or else were subjected to electrical impulses before placing in the trichloroacetic acid. Tissues were then disintegrated and the acid-soluble phosphates analysed as described by Heald (1956).

Radioactivity was measured on liquid samples in M6H counters (20th Century Electronics Ltd.), using an Ecko N529 scaler unit. All counts were corrected for background and decay. Ionophoresis records were examined with a Panax GM Monitor, Model 50 (Panax Equipment Ltd., 173 London Road, Mitcham, Surrey).

Phosphorus. This was determined as orthophosphate by the method of Berenblum & Chain (1938) as modified by Long (1943). Since this method yields the phosphate as a complex in *iso*butanol the quantity of phosphate and the radioactivity can be measured in the same solution, thus permitting a precise estimate of specific activity (cf. Ernster, Zetterstrom & Lindberg, 1950; Ennor & Rosenberg, 1952).

Alternating condenser pulses. These were supplied at 100/sec. from the apparatus of Ayres & McIlwain (1953) with a peak voltage of 18 v and a duration of 0·4–0·5 msec.

Units. The specific activity is defined as the counts/min./μg. of phosphorus. The relative specific activity is defined as the specific activity of a phosphate in the slice, such as creatine phosphate, calculated on the assumption that the specific activity of the original medium was 2×10^4. Thus in each experiment the actual specific activity of the incubation medium was determined and the quotient, 2×10^4/specific activity of the medium, used to multiply the specific activity of any phosphate of the slice. In the majority of experiments the specific activity of the medium was between 1·5 and $1·9 \times 10^4$.

RESULTS

Though the process of phosphate transport into the slice appears to be complex (Greenberg, 1952) it seemed reasonable to suppose that the amount of labelled phosphate entering would depend partly on the amount of unlabelled phosphate in the medium surrounding the tissue slice. This was found to be the case. Fig. 1 shows the specific activity found in creatine phosphate in slices, incubated either in a medium to which 6·3 mM phosphate had been added or in a medium initially free from phosphate. The specific activity was related to a solution count of 10^5 counts/min./ml., and it is seen that in the high-phosphate medium a period of some 18 min. was required for the activity of the creatine phosphate to reach a level attained in 3 min. by slices incubated in the 'phosphate-free' medium. Actually it was found that the saline initially free from phosphate had in fact accumulated orthophosphate during the preliminary incubation period. Though the quantities averaged 4·25 μmoles of phosphate/g. wet wt. of tissue over a large number of experiments, there was some variation from experiment to experiment, even though the ratio, wt. of tissue: vol. of saline, was maintained nearly constant. For this reason it was considered desirable to calculate the relative specific activities of the individual phosphates on

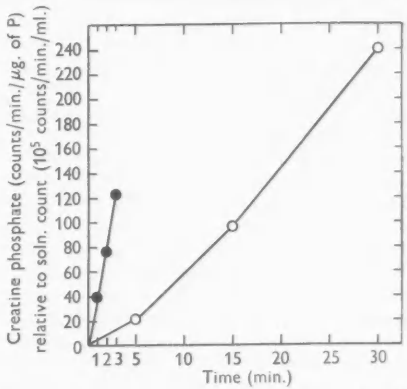


Fig. 1. Rate of incorporation of radioactive phosphate into creatine phosphate in guinea-pig cerebral slices. ●, Medium with no additional phosphate; ○, medium containing 6·3 mM potassium phosphate. Salines and conditions as in Methods.

the basis of the specific activity of the incubation medium rather than on the basis of a standard count in solution (cf. Sacks & Culbreth, 1951). By maintaining the specific activities of the incubation media reasonably constant a comparison of different experiments by simple proportion could be made. As will be shown later, this does not apply to media whose specific activities differ widely. From the above experiments it was concluded that the use of a phosphate-free saline was desirable since the short incubation time needed to build up a high activity in creatine phosphate would minimize the chance of a more even distribution of labelled phosphate taking place throughout the tissue. This would permit subsequent changes in radioactive phosphate distribution to be more easily detected.

Removal of extraneous radioactive phosphate. Calculations of the absolute rate of renewal and turnover of phosphates within a slice are possible only if the intracellular concentration of radioactive phosphate is known (Zilversmit, Enteman & Fishler, 1943). To try to obtain such a value the majority of workers have estimated the contribution to tissue radioactive phosphorus made by the extracellular phosphorus. The techniques used have been critically examined by Ennor & Rosenberg (1954), who concluded that assessments of this type are open to certain errors which have not yet been overcome. Thus in some instances the values calculated for the extracellular contribution are greater than the total actually found (Sacks & Altschuler, 1942). Also, during procedures involving either perfusion (Kalckar, Dehlinger & Mehler, 1944) or, with slices, washing in fresh saline at 0° for periods of 30 or 60 min. (Furchgott & Shorr, 1943), phosphate exchange within the tissue proceeds at a measurable rate, rendering the final results difficult of interpretation in terms of events taking place at the supposed termination of the experiment. In view of this it was decided not to try to estimate extracellular space in the cerebral slices, but to use a washing technique of brief duration, although sufficient to yield reproducible results for total tissue radioactive orthophosphate.

In Fig. 2 is shown the rate of removal of adherent radioactive phosphate when slices and holders, after incubation for 3 min. in the presence of labelled phosphorus, were rapidly transferred to 20 ml. of fresh phosphate-free oxygenated saline. It is seen that removal was virtually complete in 5 sec. Accordingly, a period of 10 sec. was chosen as a standard washing period before slices were subjected to further treatment. The constancy of the specific activity of phosphate within the tissue after washing was maintained within reasonable limits both from slice to slice and from one group of

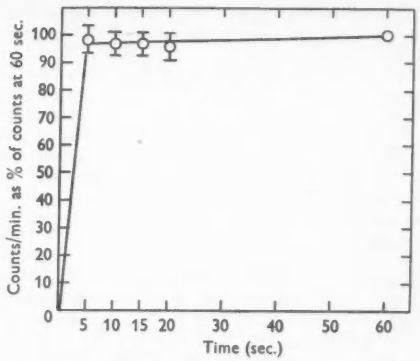


Fig. 2. Rate of removal of radioactive phosphate adhering to cerebral slices and to the tissue-holder, when washed for different periods of time in warm oxygenated phosphate-free saline. The horizontal lines on either side of the experimental points indicate the S.E.M. of six determinations. Salines and conditions as in Methods.

slices to another. Thus in five experiments using different samples of radioactive phosphate the variation of the specific activities of each group varied from 7·8 to 11·2% of the average values. Since the amount of medium carried over into the washing saline by each slice and holder was found to be $0{\cdot}42 \pm 0{\cdot}03$ (6) ml., i.e. a variation of 7·1%, the above values for radioactive phosphorus in the slices were considered to be as good as could be expected. The procedure did not affect the metabolism of the slices as judged by the levels of creatine phosphate. Thus in three experiments the levels (with standard errors) were $1{\cdot}38 \pm 0{\cdot}1$ (4) $1{\cdot}44 \pm 0{\cdot}19$ (6) and $1{\cdot}42 \pm 0{\cdot}05$ (5), all of which are normal for tissues respiring in glucose salines (McIlwain, Buchel & Cheshire, 1951; McIlwain & Gore, 1951; Heald, 1954).

Time course of incorporation of radioactive phosphate in cerebral tissue. With the above information, an attempt was made to plot the time course of radioactive phosphate distribution in the soluble phosphates with a view to selecting a point most suitable for studying the effects of applied pulses. In Fig. 3 is shown the distribution of activity between the major acid-soluble phosphates determined in extracts of guinea-pig cerebral slices incubated for periods of up to 3 min. in the presence of radioactive orthophosphate, and washed for 10 sec. and again incubated in 5 ml. of fresh saline. Although the quantities incorporated appear to be considerable, this is due to the high activity of radioactive phosphorus in the incubation medium, for in fact not more than 1% of the labelled phosphate added was assimilated after 3 min. incubation.

During the initial period incorporation was most rapid into the γ-P of adenosine triphosphate, followed closely by creatine phosphate and more slowly by the hexose monophosphate group. On removal from the radioactive incubation medium and reincubation in fresh saline all the activities fell steadily. It is of interest that the curve for the γ-P of adenosine triphosphate crossed the curve for creatine phosphate below the peak of the latter. The possible significance of this will be discussed later. The relative specific activity of the tissue orthophosphate was much above that of any other component. This must in part be attributed to extracellular phosphate not removed by washing. The fall in this, and the other phosphates, can partly be accounted for by loss of labelled phosphate to the incubation medium. Thus measurement of orthophosphate in the second incubation medium revealed a small and variable but quite definite quantity, the values and standard errors being $0{\cdot}29 \pm 0{\cdot}04$ (9) μmole/g. wet wt. of tissue after 2 min. further incubation, and $0{\cdot}384 \pm 0{\cdot}185$ (7) μmole/g. wet wt. of tissue after 5 min. further incubation. The relative specific activities $\times 10^{-3}$ of this phosphate were $2{\cdot}95 \pm 0{\cdot}95$ (9) and $3{\cdot}6 \pm 1{\cdot}0$ (7) respectively, i.e. $14{\cdot}7 \pm 4{\cdot}75$ and $18{\cdot}0 \pm 5{\cdot}0$ % of the specific activity of the phosphate in the original incubation medium. This would appear to mean that of the orthophosphate appearing in the medium after the second incubation some 80–85 % is derived from the tissue phosphates and does not arise from phosphate of the original medium adhering to the slices and being mechanically transferred. There was no general increase in the relative specific activities of the phosphates in the fraction with soluble barium salts not precipitated by ethanol. This fraction contained $1{\cdot}3$ μmoles of phosphate/g. wet wt. of tissue and reached a relative specific activity of 24·0 in the initial incubation period of 3 min. After the slice had been washed, and further incubated for 2, 5 and 10 min., the values were 21·0, 22·0 and 22·0 respectively. This fraction has been shown to contain some radioactive orthophosphate even after the ethanol precipitation (Heald, 1956), which in part accounts for the constancy of the relative specific activity. The fraction was not examined further.

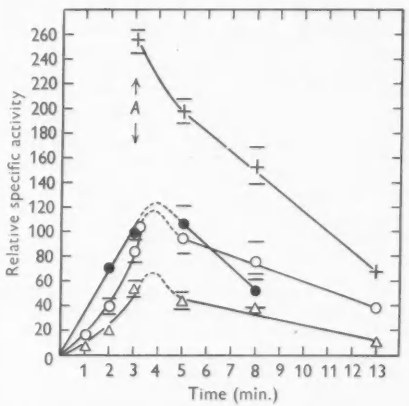


Fig. 3. Time course of incorporation of radioactive phosphate into guinea-pig cerebral slices incubated in an aminotrishydroxymethylmethane-buffered saline containing glucose. Tissues were removed at the time indicated by the arrows *A*, washed for 10 sec. in saline and re-incubated in fresh saline. +, Orthophosphate; ●, adenosine triphosphate γ-P; ○, creatine phosphate; △, hexose monophosphates. The dotted line between the points at 3 and 5 min. indicates the possible change during this interval. The horizontal lines on either side of the experimental points represent the S.E.M. values. Several of these lines have been omitted for the sake of clarity.

Effect of electrical pulses

The results obtained suggested that a suitable point at which applied pulses might show the greatest effect was 2 min. after removal of the tissue from the preliminary incubation medium. It had been shown (Heald, 1954) that the application of pulses to cerebral slices in a system similar to the one used here resulted in a rapid breakdown of creatine phosphate, which was complete within 5–7 sec. Accordingly, a period of 10 sec. was chosen as being sufficient to ensure that the rapid breakdown of creatine phosphate would be complete.

Slices from one cerebral hemisphere were incubated in the presence of radioactive phosphate, and washed and reincubated for 2 min. Pulses were then applied for 10 sec., the slice being fixed in ice-cold trichloroacetic acid during the 10th to 11th sec. Control slices from the other cerebral hemisphere were treated similarly but without applying pulses. The results of these experiments are shown in Table 1, from which it is apparent that pulses produced a marked change in the phosphates. Thus in tissue which had been electrically stimulated there was a decrease in the relative specific activity not only of the tissue orthophosphate but also of the γ-P of adenosine triphosphate, of creatine phosphate and of the hexose monophosphate group. The level of orthophosphate had risen and that of creatine phosphate had fallen, as compared with the control slices. There were also slight increases in levels of the hexose monophosphate group and adenylic acid. The probable nature of the fraction '*A*' has been discussed previously (Heald, 1956), and although values for

relative specific activity are quoted here, the possibly composite nature of this fraction and the fact that it may contain variable quantities of radioactive orthophosphate which had not been precipitated as the water-insoluble barium salt makes comparisons of the two sets of values of little use.

In these experiments the sum of the counts found in the various fractions after deionization (Heald, 1956) was almost equal to the total counts found in the original extract, the recoveries and standard errors being $99{\cdot}1 \pm 2{\cdot}2\%$ (10) for stimulated tissues and $97{\cdot}4 \pm 2{\cdot}6\%$ (6) for unstimulated tissues. These values showed that little or no radioactive phosphate was being absorbed on the ion-exchange resins. Examination of the ionophoretograms with a Panax monitor did not reveal the presence of any active area other than those estimated and listed in Table 1.

The figures in Table 1 show clearly that there was a general decrease in the relative specific activity of the phosphates in extracts of the tissues subjected to pulses. This suggested that radioactive phosphate was being transferred from acid-soluble to acid-insoluble phosphate, and accordingly determinations were made of the relative specific activity of the phosphates remaining in the tissue after trichloroacetic acid extraction. The results of these experiments are presented in Table 2, in which, in addition to relative specific activity of the residues, values are given for the relative counts found in the trichloroacetic acid extracts. It must be pointed out that these latter figures, although calculated to permit comparison between the extracts, have no precise significance as regards their magnitude. The relative specific activity of the residual tissue phosphate was in all cases greater in tissues subjected to pulses than in tissues not stimulated. Parallel with this there was a decrease in the relative total count found in the trichloroacetic acid extracts. The reasons for the variation between the individual experiments in the relative

Table 1. *Effect of electrical pulses on the distribution of radioactivity in the acid-soluble phosphates of guinea-pig cerebral tissues*

Conditions as described in Methods. Pulses: condenser pulses, 50 cyc./sec.; peak voltage: 18v; duration: 0·4 msec.; period of application: 10 sec. Values given ± S.E.M. Figures in parentheses show the number of experiments.

	With pulses		Without pulses	
Phosphate estimated	Levels (μmoles/g. wet wt.)	Relative specific activity	Levels (μmoles/g. wet wt.)	Relative specific activity
Orthophosphate	1·92 ± 0·18 (7)	160 ± 6·3 (8)	1·12 ± 0·12 (4)	199·3 ± 9·2 (7)
Adenosine triphosphate γ-P	—	43·6 ± 6·4 (9)	—	110·6 ± 13·5 (8)
Creatine phosphate	0·55 ± 0·09 (8)	40·0 ± 5·9 (7)	1·15 ± 0·12 (6)	92·0 ± 10·0 (9)
Hexose monophosphate	0·64 ± 0·065 (9)	22·6 ± 4·4 (7)	0·52 ± 0·12 (6)	42·6 ± 6·2 (9)
Adenylic acid	0·76 ± 0·15 (8)	28·5 ± 8·5 (9)	0·48 ± 0·15 (5)	44·3 ± 9·0 (6)
Diphosphopyridine nucleotide	0·39 ± 0·14 (7)	4·9 ± 1·6 (6)	0·27 ± 0·038 (5)	5·7 ± 1·2 (6)
Fraction '*A*' (Heald, 1956)	0·64 ± 0·10 (9)	70·0 ± 3·6 (8)	0·91 ± 0·09 (6)	139 ± 40 (5)

Table 2. *Effect of electrical pulses on the relative specific activities of the (tissue) residual phosphate, after extraction with trichloroacetic acid, and on the distribution of the relative counts in the extracts*

For methods see text. The relative counts/min. in the extracts are calculated as counts/min. of extract relative to a specific activity of 2×10^4 in the incubation medium. Data for Expts. 7–12 were obtained from experiments described in Table 1.

	Relative specific activity of residues			Relative counts in extracts		
Expt.	With pulses (1)	Without pulses (2)	Difference (1 – 2)	With pulses (3)	Without pulses (4)	Difference (3 – 4)
1	3·68	3·50	+0·18	—	—	—
2	3·85	2·78	+1·07	61 500	65 600	−4100
3*	9·1*	8·1*	+1·0	38 200	45 500	−7300
4	1·47	1·12	+0·35	23 800	25 500	−1700
5	1·75	1·08	+0·67	31 200	33 600	−2400
6	2·02	1·62	+0·40	32 600	38 400	−5800
7	—	—	—	15 200	19 200	−4000
8	—	—	—	24 800	23 000	+1800
9	—	—	—	16 600	19 700	−3100
10	—	—	—	31 800	33 400	−1600
12	—	—	—	28 200	36 200	−8000

* The tissues in this experiment were extracted once only with trichloroacetic acid.

specific activity of the residues is not clear. Possibly the efficiency of washing of the residues varies from experiment to experiment.

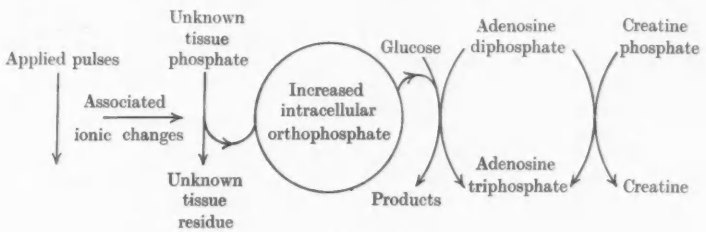
DISCUSSION

The most important of the results presented in this paper appear to be the changes taking place in the relative specific activities of both the acid-soluble and acid-insoluble phosphates on passing electrical pulses.

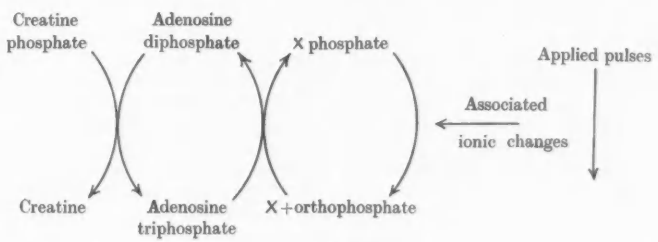
As stated in the introduction, the experiments were considered in the hope that they might indicate whether the increased orthophosphate found in the tissue after stimulation was derived from creatine phosphate. Calculations based on the data in Table 1 show that if the increase of 0·8 μmole for the phosphate/g. wet wt. of tissue was derived from the phosphate of creatine phosphate then the relative specific activity of the total orthophosphate would be 155·0. This value agrees almost exactly with the value of 160·0 found experimentally. However, a simple breakdown of creatine phosphate to creatine and orthophosphate should not result in any decrease in the relative specific activity of the residual creatine phosphate, whereas a decrease was found not only in this quantity but in the relative specific activity of the γ-P of adenosine triphosphate and in the hexose monophosphate group. Parallel with this there was a definite increase in the relative specific activity of the residual acid-insoluble phosphate.

The two most obvious interpretations of these results appear to be either that (*a*) electrical pulses release unlabelled or weakly labelled phosphate from the insoluble phosphates, possibly as orthophosphate which by entering the intracellular phosphate pool becomes available for incorporation into adenosine triphosphate and thence to creatine phosphate and orthophosphates, or (*b*) pulses promote an exchange between a phosphate such as adenosine triphosphate and a weakly labelled or unlabelled organic phosphate more closely associated with the basic changes caused by electrical pulses (McIlwain & Gore, 1953) than adenosine triphosphate itself. These two interpretations are presented in Schemes *A* and *B*.

Scheme *A* is difficult to reconcile with the data. Thus although such change would result in a decrease of the relative specific activity of the intracellular orthophosphate there is no reason why the relative specific activity of adenosine triphosphate and of creatine phosphate, already below the relative specific activity of the tissue orthophosphate, should decrease still further. Neither does the scheme offer an explanation for a decrease in the level of creatine phosphate. While the possibility must remain that interactions of the various phosphates may take place in certain localized areas, such a hypothesis is difficult to test in the experimental system described, for pulses appear to be effective only in intact nervous tissue (Kratzing, 1951; McIlwain & Narayanaswami, 1954).



Scheme *A*



Scheme *B*

On the other hand, Scheme *B* permits a reasonable interpretation of the observed data. Thus the tissue organophosphate, labelled X-phosphate, can break down to yield orthophosphate, and can be rephosphorylated by adenosine triphosphate which is in turn rephosphorylated by creatine phosphate. During this process the residual acid-insoluble phosphates containing X-phosphate would increase in relative specific activities due to transfer of the adenosine triphosphate phosphorus. The net result of this process is a decrease in the level of creatine phosphate and an increase in the level of inorganic phosphate.

The rates at which such reactions must proceed to achieve the above results within 10 sec. are well within the rates found for two phosphate transferring systems known to occur in guinea-pig cerebral tissue. Thus the rate of transfer of the phosphate of creatine phosphate to adenosine diphosphate by cerebral creatine phosphokinase is 3600 μmoles of phosphate/g. wet wt./hr. (Narayanaswami, 1952), a rate sufficient to enable the total tissue creatine phosphate to be turned over some 6–7 times in 10 sec., the period during which impulses were applied. Similarly, the adenosine triphosphatase of cerebral tissue converts adenosine triphosphate into adenosine diphosphate and orthophosphate at a rate of 2000 μmoles/g. wet wt./hr. (Gore, 1951), sufficient to split cerebral adenosine triphosphate some 5–6 times in 10 sec. It does not seem unreasonable to suppose that the increased enzymic activity of cerebral tissues brought about by applied impulses, as shown by increased respiration and lactic acid production, should also include systems of the adenosine triphosphatase type.

The proposed existence of a phosphate such as X-phosphate is supported by other evidence. Thus with cat-cerebral slices in a glucose–saline medium, Findlay, Strickland & Rossiter (1954) have demonstrated a relatively rapid incorporation of externally added radioactive phosphate into a fraction thought to be phosphoprotein, which remains in the tissue residue after extraction with trichloroacetic acid. Further, it has been shown by Fleckenstein, Janke, Davies & Krebs (1954) that frog muscle contains an unknown phosphate which, on brief electrical stimulation for 1 sec. at 0°, releases orthophosphate before any change in the levels of creatine phosphate or adenosine triphosphate can be detected.

The changes in relative specific activity of creatine phosphate, adenosine triphosphate and orthophosphate as a result of electrical stimulation permit some observations to be made regarding the inhibition of oxidative phosphorylation supposed to be brought about by applied impulses. It has been stated (Abood, Gerard & Ochs, 1952; Abood, Gerard & Tschirgi, 1952; Abood, 1954) that in systems of cerebral mitochondria supplemented with yeast hexokinase and glucose, the passage of electrical pulses from a variety of electrodes brings about a reduction in the amount of phosphorus esterified in relation to the increase in oxygen uptake. Such conclusions have been drawn on the basis of a decrease in the specific activities of the hexose monophosphate formed and of the orthophosphate in the system, though this latter was apparently heavily contaminated with up to 30% of adenosine di- and tri-phosphates (Abood & Gerard, 1952). Certain of these effects are due to artifacts (McIlwain & Narayanaswami, 1954; Chappell & Greville, 1954), as agreed by Abood (1954). Others may be capable of interpretation by an exchange mechanism of the type outlined in Scheme *B*.

Certain other points deserve mention. In Fig. 3 the curves showing the relative specific activities of creatine phosphate and of the γ-P of adenosine triphosphate intersect at a point below the peak of the creatine phosphate curve. Such a result, interpreted along the lines developed by Zilversmit *et al.* (1943) for product-precursor relationships in phospholipid biosynthesis, would suggest that the γ-P of adenosine triphosphate is not the immediate precursor of creatine phosphate. The factors involved in relationships of this type have been reexamined by Hearon (1951), who concluded that while obedience to the simple product–precursor relationship implies that one compound is a precursor of the other, the reverse is not necessarily true. Other factors to be considered in interpreting the results must include the change in saline medium during the experiments and, in addition, the fact that the differences between the points plotted on the descending curves for adenosine triphosphate and creatine phosphate are not statistically significant. It is considered that the results in Fig. 3 are consistent with the view that the γ-P of adenosine triphosphate is the precursor of creatine phosphate (cf. Ennor & Rosenberg, 1954).

Table 1 shows that the level of the hexose monophosphate group changed little in response to pulses, though the relative specific activity decreased. The probably composite nature of this group has been discussed previously (Heald, 1956), and until detailed analysis has been performed changes in relative specific activity and their magnitude cannot adequately be assessed. The small increase in quantity in the stimulated tissues may be compared with the finding of Dawson & Richter (1950) that during the application of electrical pulses to the brains of rats *in vivo* cerebral hexose monophosphate increased. Apart from the difference between the *in vivo* and *in vitro*

systems, another difference lies in the method of analysis employed. Thus, while Dawson & Richter analysed the cerebral tissues by the method of Stone (1943), the fraction designated 'hexose-phosphate' consisted of all the phosphates other than creatine phosphate, which were not precipitated by calcium acetate or hydroxide from an alkaline solution containing 10% (w/v) ethanol. This fraction is known to contain many phosphates in addition to hexose monophosphates (Stone, 1943).

A final point concerns the use of the specific activity of orthophosphate in solution as a means of calculation of the relative specific activities of tissue phosphate. It is the practice of the majority of workers to arrange that in experiments *in vitro* the quantity of orthophosphate in the medium is greatly in excess of the quantity of phosphate in the tissue, thus ensuring that such changes as may occur are minimal. In the system used in this work additional orthophosphate was absent, and it was found desirable to maintain the specific activities of the solutions nearly constant. In such a system, if the period of incubation is short, a simple proportional relationship between the specific activity of the different media and an arbitrary standard specific activity can be used as a basis for calculation. If, however, the specific activities of the media vary widely this no longer holds. Thus in collaboration with Dr A. S. C. Ehrenberg, it was found in preliminary experiments that in media in which the specific activity varied from $5{\cdot}68 \times 10^2$ to $3{\cdot}62 \times 10^4$ the relationship between the specific activity of the medium and the specific activity of the creatine phosphate in the slice was given by

$$\sqrt{c} = (0{\cdot}1m + 0{\cdot}25)t + 2{\cdot}00,$$

where c = specific activity of creatine phosphate, m = specific activity of medium $\times 10^{-3}$ and t = period of incubation in minutes.

Generally, the work described above shows that in cerebral tissues *in vitro* the rapid breakdown of creatine phosphate occurring in response to electrical pulses is accompanied by an equally rapid redistribution of the phosphates of the tissue, and the nature of the changes involved is being studied.

SUMMARY

1. A study has been made of the effects of electrical pulses applied for 10 sec. on the distribution of radioactivity amongst the acid-soluble phosphates of guinea-pig cerebral cortex.

2. In tissues exposed to pulses for 10 sec. the relative specific activity of inorganic phosphate decreased from 199·3 to 160, that of the γ-P of adenosine triphosphate from 110·6 to 43·6, and that of creatine phosphate from 92·0 to 40·0.

3. Accompanying the decrease in the relative specific activity of the acid-soluble phosphorus, the residual insoluble phosphorus of the tissues exposed to pulses showed an increase as compared with the control tissues.

4. The results are discussed in the light of a proposed exchange mechanism operating between the acid-soluble and -insoluble phosphates.

The author thanks Professor H. McIlwain for his interest and advice during the course of this work.

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The Glucose, Glycogen and Aerobic Glycolysis of Isolated Cerebral Tissues

By H. McILWAIN and MARGARET A. TRESIZE
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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The rapidity of response of many chemical processes in the brain to its excitation *in vivo* is impressive, and for adequate characterization requires study in a simpler system than the whole animal. The present paper concerns aspects of cerebral carbohydrate metabolism which have been studied in isolated tissues. Changes in the first few seconds of their excitation (McIlwain, 1951) have proved to differ qualitatively and quantitatively from the more sustained changes studied by ordinary methods over periods of minutes or hours.

Glycolysis when the brain is excited *in vivo* can reach 350 μmoles of lactate/g./hr., which is also reached in fortified homogenates, though in isolated cell-containing systems with various stimulating agents the most rapid sustained rate of glycolysis is one-third or less of this value (see McIlwain, 1955). By the use of apparatus permitting rapid handling of the isolated tissue (Heald & McIlwain, 1956) a brief burst of rapid glycolysis has now been observed *in vitro*. Associated studies have concerned glucose and glycogen as potential sources of the lactate, and have involved examining the distribution of glucose and lactate between tissue and fluid during *in vitro* experiments.

EXPERIMENTAL

Tissues

Most experiments employed adult guinea pigs weighing 350–500 g., of either sex and fed on a mixed diet. The cerebral cortex was prepared as slices 0·35 mm. thick and weighing about 120 mg., as described by McIlwain (1951). Three slices were cut successively from first one and then the other hemisphere, and the slices were handled throughout in the order in which they were cut. Their glycogen content varied with this order, as is described below. After cutting, slices were floated to the experimental saline, drained from excess of it, weighed on a torsion balance and placed in separate experimental vessels.

Incubation. Salines, based on those of Krebs, are described by Rodnight & McIlwain (1954). Respiration and acid formation in some experiments were measured in orthodox manometric apparatus, and the tissue was subsequently removed promptly for analysis by picking it from the vessel with a mounted, bent wire.

The majority of experiments, which involved exposing the tissues to changed experimental conditions for brief periods, were carried out in the apparatus of Heald & McIlwain (1956). Here the tissues (control and experimental) were held between grids of silver wire, in 5 ml. of the experimental saline in a thermostat at 37·5°. Incubation usually began 15 min. after the death of the animal. In some experiments alternating electrical condenser pulses were applied to the silver grids from the generator of Ayres & McIlwain (1953).

Transference of tissues after incubation. The slice was lifted in its holder from the fluid, leaving the tip of the holder touching for 2 sec. the side of the beaker which had contained it. The slice was then released to a fixing or other fluid, this transfer requiring only about 0·25 sec. During the period of 2 sec. it was considered that metabolic conditions in the tissue were not altered by the partial draining, for many metabolic characteristics of such tissue remained unchanged after much longer periods of incubation with minimal fluid (Rodnight & McIlwain, 1954). If the terminal period of an experiment included the application of pulses, this was continued during the draining.

The fluid transferred with slices of 120 mg. drained in this way was found by weighing to be $0{\cdot}26 \pm 0{\cdot}06$ g. (S.D.; 6 expts.). When the concentration of substance being determined was less in the fluid than in the tissue it was found satisfactory to calculate from this value the amount of substance transferred. In other cases the tissue was briefly rinsed, as described below. The quick-transfer apparatus was arranged to contain, in one or more of its 30 ml. beakers, 15 ml. of the medium used in incubation but which lacked the substance being determined. This medium was kept oxygenated and the slice was released from its holder to this medium, picked from it with a mounted, bent wire and put in the fixing agent. Rinsing and transfer then occupied about 3 sec., and pulses could not be maintained during this time.

Administration of general depressants and iodoacetate. For some of the experiments of Table 2, tissues were taken from guinea pigs which had been treated in one of the following ways. Pentobarbitone was injected intraperitoneally as a solution (Abbott Laboratories) of 60 mg./ml. in aqueous ethanol and propylene glycol, normally at 1 ml./kg. Reflexes were lost after about 10 min. The brain was removed after 20 min. A mixture of $N_2O + O_2$ (80:20, v/v) was delivered from a dentist's apparatus to a funnel over a guinea pig; after about 60–90 sec., when the animal was lying and breathing heavily and steadily, the tissue was removed. A *cyclo*propane–O_2 mixture was adjusted to produce similar effects, and administered in the same way. For as long as possible during their preparation, the tissues in N_2O and *cyclo*propane experiments were kept in the corresponding gas mixture, which was also bubbled through the salines in which the tissue was handled.

Iodoacetic acid (as a solution of the Na salt at pH 7·3 containing 100 mg. of the acid/ml.) was administered intraperitoneally at 4 ml./kg. Abnormal behaviour began at 8–10 min. after injection, and animals were stunned at 12–14 min. when convulsions appeared to be beginning.

Determination of glycogen and lactate

When in previous work glycogen and lactate have both been estimated in cerebral tissues, separate samples have been taken for the two determinations (e.g. Kerr, Hampel & Ghantus, 1937; Klein & Olsen, 1947; Gurdjian, Webster & Stone, 1949). As the present investigations concerned the concomitant change or lack of change in the two substances in small portions of tissue, a method was devised for determining them both in the same sample. Ethanol was chosen for the initial fixing of the tissue as it appeared to penetrate it quickly and leave it in a suitable physical form, still easily releasable from the holders, which were not damaged by the ethanol.

Method. The tissue was usually a slice weighing 120 mg., and six such specimens were handled successively at brief intervals. Each, in a quick-release holder, was allowed to drain for 2 sec. and released into 3 ml. of absolute ethanol in a 30 ml. squat beaker. After 3 min. it was washed from the beaker with the ethanol to a 15 ml. test tube and broken with a glass rod. The tube was centrifuged 10 min. later, and the ethanolic extract was returned to the same beaker; the residue in the tube was rubbed in a further 2 ml. of ethanol with the same rod, and after centrifuging the second extract was added to the first.

Glycogen was determined in the residue in the tube, which was well drained from ethanol on each occasion. Ethanolic KOH (1 ml. from a mixture of 2 vol. of ethanol and 1 vol. of 60%, w/v, aqueous KOH) was added and the estimation completed according to LeBaron (1955).

The beaker containing the ethanol extracts was placed with others from the same experiment over $CaCl_2$ in a desiccator which was evacuated and left at 3° for 6 hr. or more. The residue was then rubbed up with 5 ml. of 2% (w/v) $CuSO_4, 5H_2O$ and lactic acid determined according to Barker & Summerson (1941).

Control experiments. Barker & Summerson (1941) observed that ethanol did not interfere in the lactate determination. We found that two specimens of absolute ethanol gave no more than 0·2 μmole of apparent lactate/ml. when added as such to standard lactate solutions, and had no effect after evaporation in the manner described above, which also did not lead to loss of lactate. The extraction removed 98% of added lactate and was not facilitated by acidifying.

Tissue glucose

Tissue fixed in 95% or absolute ethanol was found to yield only part of its glucose to the ethanol. In the instances in which glucose was determined, separate slices were used. These were released to 15 ml. of glucose-free oxygenated medium at 37·5°, and transferred with a wire to 1 ml. of 0·1N-$Ba(OH)_2$ in a 10 ml. tapered centrifuge tube, and immediately ground with a glass pestle shaped to fit the tube. Zinc sulphate soln. (1 ml., adjusted to leave the solution just pink to phenolphthalein) was added and the determination completed according to Nelson (1944).

RESULTS

Lactic acid distribution and formation

Content in normal tissue and fluid. The fluids in which the slices were immersed contained initially no lactic acid. The tissue immediately after death of the animals contained about 9 μmoles/g., but the greater part of this was lost to the fluids used in preparing the tissue. The lactate content of the slices of tissue after incubation was found to be relatively uniform (Table 1), independent of the order in which the slices were cut or of the depth in the cerebral cortex which the tissue had formerly occupied. Thus after 1 hr. at 37° the mean lactate concentrations in the second and third slices cut from a hemisphere were 103 and 88% of that in the first; in the second hemisphere, that of the second and third slices combined was 96% of that in the first. None of these values differed significantly from 100%. On the other hand, experiments carried out some time apart with different animals and different batches of media gave significantly different results. Contributing to the value of 1·51 ± 0·46 μmoles/g. of Table 1 were values for successive slices from the hemisphere of one animal, of 1·24, 1·42, 1·25, and from another, of 1·62, 1·91, 1·94.

Table 1. *Lactic acid of cerebral tissues after incubation in salines of different glucose levels*

Slices of cerebral cortex weighed 100–120 mg. Some, giving the data at zero time, were without contact with fluid. Others were in quick-transfer electrodes in 5 ml. of oxygenated, phosphate-buffered glucose–salines, and were fixed in ethanol after the periods stated. S.D and number of observations are given in one case; other results are averages of duplicates which differed by less than 15%.

Initial glucose of fluid (mM)	Time of incubation (min.)	Lactic acid: In fluid (μmoles/ml. of fluid)	Lactic acid: In fluid (μmoles/g. of tissue)	Lactic acid: In tissue (μmoles/g. of tissue)
(No fluid)	0	—	—	8·62
10	30	0·42	20·2	1·36
10	60	0·62	23·7	1·51 ± 0·46 (15)
10	120	0·60	31·4	0·82
2·5	60	0·61	22·8	1·22
1·0	60	0·24	10·9	0·97

The concentration of lactate after incubation was in all cases higher in the tissue than in the surrounding fluids (Table 1). Thus under the conditions most frequently employed, in a fluid with 10 mM glucose for 1 hr., lactate in the tissue was at about 2·5 times its level in the fluid. This difference was greater earlier in the experiment, when the rate of formation of lactate was greater, and fell further after incubation for 2 hr. The rates of formation in these experiments were about 20–30 μmoles of lactate/g./hr. and are typical of tissue prepared and handled in this way (McIlwain & Buddle, 1953; Rodnight & McIlwain, 1954). The total lactate formed after incubation for 1 hr. fell only a little when the initial glucose of the fluid was lowered from 10 to 2·5 mM, but fell to less than half with 1 mM glucose, as noted under other experimental conditions (McIlwain, 1953). In the present experiments the volume of fluid was about 40 times that of the tissue, so that, in spite of the distribution described, the fluid contained 90–97 % of the total lactate formed.

Electrical pulses and lactate formation. The pulses sharply increased the tissue lactate. Fig. 1 shows this in tissues after incubation for 1 hr., in response to pulses of a potency just greater than previously found necessary for maximum metabolic effect. In 7 sec. the tissue lactate had increased by over 50 %. By contrast, changes in the lactate of the fluid during the whole 80 sec. of such experiments were small.

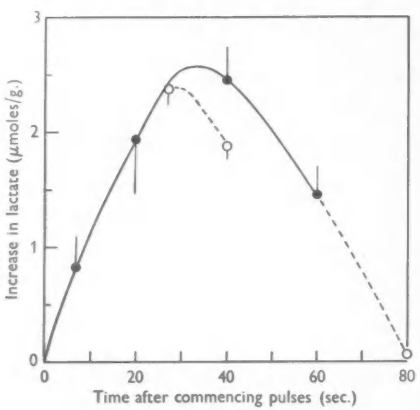

Fig. 1. Changes in the lactic acid of guinea-pig cerebral cortex after applying electrical pulses for the periods indicated. Prior to application of pulses all tissues had respired in phosphate salines with 10 mM glucose for 1 hr. Condenser pulses were alternating, of peak potential 10 v, time-constant 0·8 msec., and at 100/sec. Continuous lines and filled-in points (●) give the change with pulses applied to the moment of fixation; open points (○) and broken lines refer to experiments in which the pulses, applied until the preceding filled-in point, were followed by a period without pulses before the tissue was fixed. Vertical lines extend from the points for a distance corresponding to the S.D., described more fully in the text, and derived from six tissue specimens.

Each experimental point of Fig. 1 gives the mean difference between two groups of slices from the same one or two experimental animals. The filled circles give the differences between slices to which no pulses have been applied and others with pulses for the periods indicated. The standard deviations quoted in Fig. 1 refer to these differences, all of which are highly significant (giving t values corresponding to levels of significance of less than 0·01). The open circles (Fig. 1) give differences between slices allowed to metabolize for a few seconds after pulses ceased, and others, which after pulses for the same duration in the same experiments were fixed immediately pulses ceased. Again the mean differences were significant at the 0·01 level except that, 20 sec. after applying pulses for 20 sec., the lactate of the tissue was not appreciably different from its value when the pulses ceased.

The initial rate of lactate formation was remarkably high, being 420 μmoles/g./hr. This, however, lasted for only about 20 sec., after which, even if pulses are continued, lactic acid at first increased more slowly and then declined. The slower increase may simply reflect loss from tissue to solution. After 60 sec. application of pulses fluids averaged 22·7 μmoles of lactate/g. of tissue, while without pulses values were 21·2. The difference of 1·5 μmoles/g. is nearly equal to that of Fig. 1 between the maximal lactate of the tissue and its content at 60 sec., but had a P value of 0·1. It is naturally more difficult to measure changes of a few μmoles of lactate in the fluid which contains much more than does the tissue. Decline in lactate on continuing the application of pulses from 20 or 40 to 60 sec. appears, however, to reflect a decrease in its formation.

When pulses were stopped before lactate formation was maximal, the tissue lactate continued to rise for several seconds. When pulses were stopped at a time when lactate was already falling, the fall also continued (Fig. 1). There is thus an interesting inertia in the metabolic response to the pulses. On the other hand, the commencement of the change was prompt, the rate during the first 7 sec. being higher than in subsequent periods.

Glycogen

Normal cerebral glycogen of about 4–6 μmoles of hexose/g. of fresh tissue falls rapidly when the brain is removed and sectioned for metabolic experiments (Kerr, 1936; see Table 2). LeBaron (1955) showed that the glycogen could be restored to such sections during 4 hr. respiration in glucose salines. The present experiments commenced with attempts to

obtain this level of glycogen in a shorter time, so that the effects of agents upon it could be more readily studied.

Attempted preservation of 'native' glycogen. It was easy to preserve a higher level of glycogen in cerebral tissues by administering general depressants before removing the brain (Table 2). Pentobarbitone was more effective than nitrous oxide or *cyclo*propane; these tend to be lost from the animal while the brain is being removed and from the tissue during its preparation, though the tissue was as far as possible handled in their presence. Administration of iodoacetate also enabled a higher level of cerebral glycogen to be preserved in the separated tissue. In all these instances the glycogen of the cerebral cortex was found to be unevenly distributed, the outer part of the hemispheres, quoted first in Table 2, being appreciably richer than the inner parts. A similar trend in glycogen level of the tissue after incubation was also found, in accordance with LeBaron (1955).

Curiously, it was not found possible to preserve the glycogen of tissues from anaesthetized animals during subsequent *in vitro* metabolism. The tissue of column 2 of Table 2 had already been prepared for metabolic experiments by cutting it into sheets 0·35 mm. in thickness; yet when these were put into oxygenated glucose salines their glycogen fell in 10 min. to 15–50% of its previous value. At least 10 min. is required under these conditions for other tissue constituents to reach stable levels, and the tissue was not therefore examined at shorter intervals. After incubation for 30 and 90 min., the glycogen in tissue from anaesthetized animals had reached levels no higher than those of tissue from animals stunned without anaesthesia, when the initial glycogen levels were low. Even when the tissue was kept in the presence of a depressant by adding the drug to the saline, the glycogen proved unstable. As the presence of a depressant was undesirable in subsequent experiments, the volatile agents nitrous oxide and *cyclo*propane were chosen, and were removed from medium and tissue after a few minutes at 37° by changing from, for example, a *cyclo*propane–oxygen mixture to pure oxygen.

Resynthesis of glycogen. Attempts to accelerate the resynthesis of glycogen have been only partly successful. Provision of lactate as well as glucose, and of glucose at higher levels, was without effect, but citrate and glutamate decreased the synthesis. Glucose 1-phosphate (5 and 20 mM; we are indebted to Roche Products for this material)

Table 2. *Glycogen in guinea-pig cerebral slices before and after incubation in different salines*

In all cases the brain was removed as rapidly as possible and the cerebral hemispheres were prepared as though for a metabolic experiment. This was done by opening the hemisphere at the lateral ventricle and cutting three successive slices from it. The three glycogen values quoted refer to these slices in the order in which they were cut, the first being from the outer part of the hemisphere. Values in the second column refer to tissues fixed immediately after weighing, about 7 min. after death of the animal; and in the fourth column to tissues fixed within a sec. after removal from the incubation medium. Glycogen was determined according to LeBaron (1955) without preliminary fixation with ethanol. Incubation fluids: see text.

Before incubation		After 30 min. (or 10 min.*) at 37°	
Preparation of animal	Glycogen in successive slices (μmoles of hexose/g.)	Saline	Glycogen in successive slices (μmoles of hexose/g.)
Pentobarbitone	4·85, 3·33, 2·92	Phosphate	1·11, 0·65, 1·03
Pentobarbitone	3·80, 2·22, 1·01	Bicarbonate	1·17, 0·55, 0·60
Pentobarbitone	—	Serum–bicarbonate (1:3)	1·02, 0·54, 0·47
Pentobarbitone	—	Glutamate–bicarbonate	0·55, 0·31, 0·22
Nitrous oxide	2·23, 1·83, 1·43	$N_2O + O_2$–phosphate	— 0·31, 0·33
Nitrous oxide	—	$N_2O + O_2$–phosphate	1·12*, 0·31* —
*cyclo*Propane	3·33, 1·89, 1·69	*cyclo*Propane + O_2–phosphate	— 0·35, 0·33
*cyclo*Propane	—	*cyclo*Propane + O_2–phosphate	1·07,* 0·37* —
Iodoacetate	2·96, 1·22, 1·12	—	—
		After 90 min. at 37°	
Stunned	0·67, 0, 0·37	Phosphate	1·8, 1·26, 1·18
Stunned	0·03, 0·56, 0·59	Bicarbonate	2·16, 0·92, 1·47
Stunned	—	Citrated human plasma	0·44, 0·17, 0·24
Stunned	—	Guinea-pig serum	— 1·24, 1·62
Stunned	—	Liver extract–bicarbonate	1·65, 1·42 —
Stunned	—	Phenobarbitone (0·3 mM) phosphate	1·59, 1·47 —
Pentobarbitone	4·09, 1·82, 1·44	Phosphate	1·59, 1·24, 1·02
Pentobarbitone and exsanguination	2·41, 1·03, 0·99	—	—

yielded less glycogen and lactic acid than did glucose. Different fluid volumes and the addition of homologous sera, of liver extracts and slices, and of fluids in which slices of liver or brain had been incubated were not advantageous, but in some cases decreased the glycogen synthesized by the cerebral tissue.

During the course of the present experiments, however, the glycogen determined in the tissue after incubation for 1 hr. in glucose saline has been approximately doubled by apparently small changes in technique. The first lay in preparing the tissue rapidly. It was normally incubated 15 min. after death of the animal. That speed of preparation is important is shown by the finding (Table 3) that slices from the second hemisphere to be cut were consistently lower than the first in glycogen level after incubation. The second modification consisted of rapidly transferring the tissue to ethanol after incubation. When so determined, the glycogen of the tissue reached about half its original level after respiration for 1 hr. Glucose provided at 10 or 2·5 mM appeared to be in excess, for glycogen levels were then similar, reproducible, and higher than

Table 3. *Glycogen in cerebral cortex after 1 hr. at 37°*

Guinea pigs were stunned, and the brain was removed; any one of the hemispheres taken and three slices were cut successively from it, weighed, and placed in holders in saline of the glucose level stated. After 1 hr. at 37° slices were transferred to ethanol for determining glycogen and lactate. The second hemisphere was treated similarly as soon as slices had been cut from the first. Values with 2·5 and 1 mM glucose are for slices from first and second hemispheres. Glycogen levels are averages followed by their S.D. and number of observations.

	Glycogen (μmoles of hexose/g.)			
	In 10 mM glucose			
Slice (order of cutting)	Of 1st hemisphere	Difference, 1st – 2nd hemispheres	In 2·5 mM glucose	In 1 mM glucose
1st	2·35±0·16 (5)	0·14±0·06 (5)	2·22±0·17 (6)	1·94±0·16 (4)
2nd	2·08±0·26 (5)	0·12±0·08 (5)	2·00±0·56 (6)	1·57±0·1 (4)
3rd	1·80±0·13 (5)	0·18±0·08 (5)	1·67±0·15 (6)	1·46±0·08 (4)
All	2·05±0·9 (15)	0·15±0·08 (15)	1·95	1·65

Table 4. *Stability of the glycogen of guinea-pig cerebral cortex*

Each line gives results from six or twelve slices from one or two guinea pigs; half the slices in each experiment were incubated under the changed conditions described in the first column. Slices were prepared and incubated as described in Table 3 and were in oxygenated salines in 10 mM glucose except when stated otherwise. Pulses were applied after 1 hr. at 37° and recovery periods were the intervals between stopping pulses and fixing the tissue. The significance of the differences induced by changed conditions was assessed by the t test but is not quoted when the significance is obviously low. In the last two experiments N_2 was blown vigorously through the saline after the incubation; some seconds were involved in displacing the oxygen but the time was not determined. Insulin (British Drug Houses, A.B. 40) was at 0·5 unit/ml.

	Difference in glycogen content: slices of 1st – those of 2nd hemisphere (μmoles of hexose/g.)		
Changed conditions or applied agents*	Agent to 1st hemisphere	Agent to 2nd hemisphere	P
None (see Table 3)	0·15±0·08		
Pulses for 7 sec.	0·33	−0·02	>0·1
Pulses for 20 sec.	0·18	0·22	—
Pulses for 60 sec.	—	0·12	—
Pulses for 20 sec.; recovery, 7 sec.	—	0·05	—
Pulses for 20 sec.; recovery, 20 sec.	—	0·05	—
Pulses for 60 sec.; recovery, 20 sec.	—	0·07	—
Insulin	0·15	0·25	—
Pulses for 20 sec.; insulin	0·34	−0·06	<0·01
Pulses for 60 sec.; insulin	0·11	0·23	—
Pulses for 60 sec.; recovery 20 sec.; insulin	0·21	0·31	—
In mM glucose; pulses 20 sec.; recovery, 7 sec.	0·11	−0·17	>0·1
In mM glucose; pulses 60 sec.; recovery 7 sec.	−0·01	0·13	—
In 2·5 mM glucose; insulin	−0·04	0·11	—
In 2·5 mM glucose; pulses 20 sec.; recovery 7 sec.; insulin	0·09	0·36	—
Nitrogen for 1 min.	−0·12	0·17	0·05
Nitrogen for 5 min.	−0·59	0·63	<0·01

* Those named last in each line constitute the difference which is assessed in cols. 2–4.

with 1 mM glucose (Table 3). The resynthesis is thus sensitive to about the same range of glucose concentrations as is the formation of lactic acid.

It was of especial interest to determine the glycogen level of the tissue during the rapid changes in lactate recorded in Fig. 1. The glycogen proved remarkably stable under these conditions. Electrical pulses applied for different periods up to 1 min. caused no significant change in glycogen level (Table 4), though in this time lactate rapidly rose and fell by amounts nearly equivalent to the total glycogen of the tissue. In other experiments the tissue was fixed not immediately after pulses had been applied but after a brief interval of recovery after their passage. This might correspond more closely to situations in which large glycogen changes have been reported *in vivo* (Chance, 1951). However, *in vitro* no appreciable change was found either with excess (2·5 and 10 mM) or with a sub-optimum level (1 mM) of glucose. Insulin was included in several experiments (Table 4) with the intention of causing greater lability in the glycogen, but was without effect. An instance was found (Table 4) in which the presence of insulin during incubation for 1 hr. followed by pulses for 20 sec. significantly increased the glycogen of the tissue; the change was small.

Anoxia for periods of a minute or more caused loss of glycogen.

Glucose

As electrical pulses increase the lactate of cerebral tissues without appreciable effect on glycogen, glucose is the most likely source of the lactate. The hexose phosphates of the tissue appear unlikely as net source, though presumably they are intermediates, for their quantity is inadequate and they slightly increase rather than decrease in amount under conditions similar to those of the present experiments (Heald, 1956), and also on stimulation *in vivo* (Dawson & Richter, 1950).

The glucose content of sliced cerebral tissues as ordinarily prepared but handled in glucose-free salines was low, being about 0·3 μmole/g.; the initial 3 or 4 μmoles/g. in the brain *in vivo* presumably contributes largely to the lactate found in the tissue *post mortem* (p. 251). After respiration for 1 hr. in phosphate salines, the glucose of the tissue rose, but remained somewhat below the level of the saline. Thus with initially 10 mM glucose, which had fallen to 9·5–9·7 mM after 1 hr., the tissue glucose was 7·3 μmoles/g. The distribution of glucose *in vitro* (Table 5) was thus rather more in favour of the tissue than obtains *in vivo*, when tissue glucose is some 36 % of the plasma level (Klein & Olsen, 1947). This may be due to the presence of more extracellular fluid in the tissue examined *in vitro*. The distribution was notably different from that of lactate, which was at higher level in the tissue, presumably reflecting the utilization of glucose and formation of lactate in the tissue itself.

Table 5. *Glucose in slices of guinea-pig cerebral cortex*

Slices prepared and handled as described in Table 1 respired for 1 hr. in oxygenated phosphate salines of the glucose content noted. Some were then rapidly released in glucose-free saline and immediately transferred to 0·1 N-$Ba(OH)_2$. To others, pulses were applied as indicated, immediately before transfer. Mean values with S.D. and numbers of observations are quoted in some cases.

Initial glucose (mM)	Time pulses were applied (sec.)	Glucose (μmoles/g.)
10	0	7·31 ± 0·35 (5)
10	20	6·44, 6·62
10	60	5·05, 6·14
2·5	0	1·30 ± 0·09 (5)
2·5	20	0·35, 0·45
2·5	60	0·29, 0·21
1·0	0	0·19 ± 0·02 (3)

Nevertheless, with each level of glucose examined, that found in the tissue was adequate to yield the additional lactate produced by pulses. That the glucose actually did fall as lactate rose is shown in Table 5. The decrease in 20 sec. was about 0·9 μmole/g., and only a further 0·2–0·5 μmole was lost in a further 40 sec. Thus the change, like that in lactate, ceases before glucose is exhausted.

DISCUSSION

The restraint normally imposed on metabolism in cerebral tissues is emphasized by the results shown in Fig. 2, which summarizes some of the present and earlier findings. Electrical pulses for a few seconds can induce lactate formation at 420 μmoles/g./hr., or over 20 times the resting rate, with corresponding loss of glucose. Formation of inorganic phosphate during the first 6 sec. of pulses is also at about 450 μmoles/g./hr. (Heald, 1954; its maximum rate during part of this time may reach 800 μmoles/g./hr.) but then ceases; the high rate of lactate formation continues for 20–30 sec. If a continuing tendency of the continuous pulses to produce phosphate is assumed, the stability of phosphate levels after 6 sec. could be accounted for by the expected esterification of 1 mole of phosphate/mole of lactate formed.

After 30 sec. with pulses, the tissue lactate falls markedly and that of the fluid rises correspondingly. The maintained rate of glycolysis is only one-fourth or one-fifth of the maximum rate. Nevertheless, inorganic phosphate does not rise at this point, although sufficient creatine and adenosine

phosphates remain in the tissue to yield 2 or 3 μmoles/g. of inorganic phosphate. Presumably either the effect of the pulses in this respect has become less, although they are still applied at the same intensity, or other phosphorylating processes have accelerated. Respiration is in fact known to increase with pulses to the extent of about 60 μmoles/g./hr., which is probably sufficient to esterify 360 μmoles of phosphate/g./hr., or approximately the deficit left when the level of glycolysis falls.

Respiration rises within a minute or two; whether it has partly replaced glycolysis at 15 sec. and completely at 30 sec., as might be suggested by the decreasing rate of lactate accumulation seen in Fig. 2, is not known. In sympathetic ganglia, respiration increases on stimulation with a time constant of about 1 min. (Larrabee & Bronk, 1952). If in general this picture of a very prompt glycolytic response succeeded by a respiratory one is correct, reason can be seen for the tissue content of glycolytic and respiratory systems being such as to give approximately equal yields of labile phosphates in a given time. This equality could also be important anaerobically. Formation of lactate even by the brain *in vivo* is not, however, only an anaerobic process, for lactate comes from the brain in venous blood still 62 % saturated with oxygen (Gibbs, Lennox, Nims & Gibbs, 1942): conceivably this is produced as in the experiments of Fig. 2 with increase of activity in a part of the brain. That the energy yield of glycolysis subserved some qualitatively different role from that of respiration was suggested earlier (McIlwain, 1953) on the basis of quite different evidence.

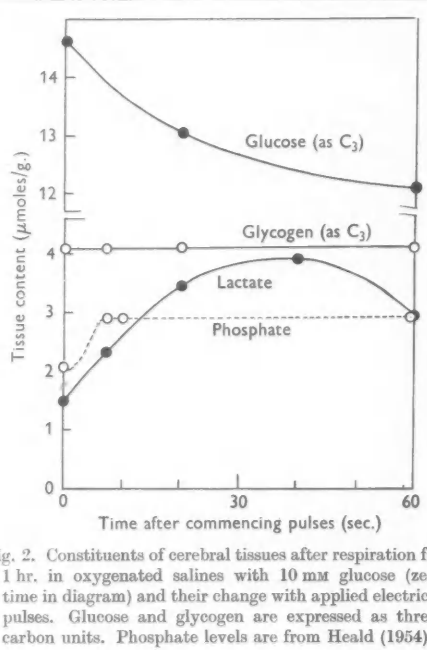

Fig. 2. Constituents of cerebral tissues after respiration for 1 hr. in oxygenated salines with 10 mM glucose (zero time in diagram) and their change with applied electrical pulses. Glucose and glycogen are expressed as three-carbon units. Phosphate levels are from Heald (1954).

SUMMARY

1. The glucose of cerebral tissues fell rapidly on excision, but was restored when the tissue respired in glucose saline; the level in the tissue remained below that of the saline.

2. The glycogen of the tissue, also rapidly lost on excision, was about half-restored after respiration for 1 hr. in glucose salines. General depressants *in vivo* preserved much of the glycogen from loss on excision, but loss nevertheless occurred on beginning respiration at 37°. Resynthesis was similar in 2·5 and in 10 mM glucose but less in 1 mM. After resynthesis, glycogen fell promptly when oxygen was excluded, but insulin and brief application of electrical pulses had little if any effect on its level.

3. The lactate of the tissue which had greatly increased *post mortem*, on respiration *in vitro* fell to levels akin to those *in vivo*, but remained greater than in a surrounding saline.

4. When electrical pulses were applied to the tissue, its glucose began within a few seconds to be converted into lactate. Conversion was at the rate of 400 μmoles of lactate/g./hr. for the first 20 or 30 sec., but greatly slowed after about 40 sec. of pulses. If pulses were stopped while the tissue lactate was still increasing, the increase continued for some seconds.

5. The maximal transitory rates of formation of lactate and of inorganic phosphate on applying pulses to the tissue were similar, and were also close to that of the formation of labile phosphates potentially associated with the observed increase in respiration. Lactate formation with continued pulses fell before glucose or phosphates were exhausted, and it is suggested that an initial momentary glycolytic response to excitation is largely replaced by a subsequent, sustained, increase in respiration.

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Anaerobic Glycolysis of Cerebral Tissues and a Second, Electrically-induced, Metabolic Defect

By H. McILWAIN
Department of Biochemistry, Institute of Psychiatry (British Postgraduate Medical Federation, University of London), Maudsley Hospital, S.E. 5

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Electrical pulses increase aerobic metabolism by separated cerebral tissues in fashions similar to those resulting from application of comparable pulses to the brain *in situ*. Linkage between electrical stimulus and metabolic response is thus open to study in the isolated tissue and previous investigations suggested an outline of such linkage (McIlwain & Gore, 1953). With pulses, several reactions are accelerated to rates beyond those of restoring systems; creatine phosphate, a major reserve of metabolically available energy, falls, but ordinarily is probably being resynthesized continually as a result of glucose oxidation; respiration increases. With the intention of characterizing other components of linking systems, pulses were in earlier studies applied aerobically to tissues without added oxidizable substrate, and their effects then found not to be immediately reversible, though with added substances a partial return to normal was achieved. In the present experiments, pulses have been applied to tissues in the presence of glucose but absence of oxygen. A second metabolic defect has so been induced, and observations have been made on its nature and means of antagonizing it.

Under the anaerobic conditions chosen for the present study the main overall reaction brought about by the tissue is glycolysis yielding lactic acid. This is already known to be affected by agents germane to these investigations, and this knowledge has been extended in the present study.

EXPERIMENTAL

Tissues and fluids

Most tissues were from guinea pigs stunned by a blow on the neck, and were prepared as slices of defined thickness and position as described by McIlwain (1951). In some cases the slices were cut to fragments as follows. After weighing (60–70 mg.) they were placed in 0·5–0·8 ml. of the experimental saline and about ten cuts made through the suspended tissue with fine dissecting scissors, yielding about thirty fragments which were transferred to the experimental vessel with a 'shovel' of silver gauze mounted in a small handle. Chopped tissue was that described by McIlwain & Buddle (1953). Tissue from rats, or tissue obtained neurosurgically from man (I am greatly indebted to Mr Murray Falconer, Guy's–Maudsley Neurosurgical Unit, for the latter), was treated similarly.

The phosphate–saline was that of Rodnight & McIlwain (1954) usually with glucose at 10 mM. In the bicarbonate–saline $NaHCO_3$ (26 mM) replaced Na_2HPO_4; equilibration was with N_2+CO_2 (95:5, v/v) or O_2+CO_2 (95:5, v/v). The dry weight (105°) of tissues as used in the present studies was found to be $15\pm0{\cdot}2\%$ (8) (standard error and number of observations) of the 'wet weight' after draining on a glass surface at room temperature. Because of differences in glycolysis in the first slice cut ('outer') and the second and third cut from a hemisphere ('inner') these were examined separately and gave: outer, $15{\cdot}4\pm0{\cdot}3\%$ (4); inner, $14{\cdot}5\pm0{\cdot}3\%$ (4).

Manometric and electrical arrangements

Pulse generators and types of vessels were those previously described or referred to by Ayres & McIlwain (1953). Vessel and electrode arrangements were tested at frequent intervals throughout the experiments to ensure freedom from changes in gas pressure or in lactate, not dependent on tissue metabolism; such control experiments under aerobic conditions have been described at some length by McIlwain (1951) and by Narayanaswami & McIlwain (1954). The following additional information, more specifically related to anaerobic conditions, was obtained during the present experiments and is summarized in Table 1.

The majority of experiments of the present series were carried out under the conditions of the first four experiments of Table 1. As will be seen, they were free from any progressive change in gas pressure or in lactate which might simulate the metabolic events measured in the

Table 1. *Testing for effects of pulses independent of tissue metabolism*

Experiments were at 37°; CO_2 when stated as present was at 5% (v/v). Pulses were alternating, of exponential voltage-time relationship, and obtained by charge and discharge of condensers collectively at 100/sec. In all experiments, the application of pulses caused a small volume change, of about 0·1 μl., which was reversed when pulses were switched off, and was previously shown (McIlwain, 1951) to be due to heating the vessels by a few hundredths of a degree by the pulses. Bicarbonate–glucose was used unless stated otherwise.

Electrode type (Ayres & McIlwain, 1953)	Metabolic conditions	Pulse: Peak potential (v)	Pulse: Time constant (msec.)	Result (progressive gas changes in μmoles/hr./vessel)
H, silver grid	N_2+CO_2	7–18	0·2–0·4	None*
	O_2+CO_2	7–15	0·3–0·4	None*
E, gold ring	N_2+CO_2	10–15	0·2–0·4	None*
	O_2+CO_2	10–18	0·2–0·4	None*
	3 mM Cysteine; O_2+CO_2	10	0·3	Gas absorption, 2
	—	15	0·4	Gas absorption, 4
	3 mM Cysteine, O_2; phosphate–glucose	15	0·4	Gas absorption, 8
	5% Yeast extract; N_2+CO_2	18	0·4	Gas evolution, 2
	0·8% Yeast extract, N_2+CO_2	18	0·4	Gas evolution, 0·4
	N_2+CO_2; yellow P	18	0·4	Gas evolution,* 4†
E, molybdenum ring	N_2+CO_2; yellow P	18	0·4	None*

* No apparent lactate when examined by the method of Barker & Summerson (1941).
† This change occurred also in the absence of glucose.

present experiments. However, under other conditions artifacts were found. Thus, if anaerobic conditions were maintained with yellow phosphorus in the centre well, application of pulses to electrodes of electrodeposited gold resulted for unexplained reasons in gas evolution from bicarbonate solutions in equilibrium with N_2+CO_2. This was not observed in vessels otherwise similar but with molybdenum electrodes; such electrodes were therefore employed in a few experiments in which strictly anaerobic conditions with yellow phosphorus were desired. Several added substances were without effect on gas-pressure changes under the conditions of the first four experiments of Table 1, but instances have been reported previously (Lewis & McIlwain, 1954), and others are included in Table 1, in which added substances cause such changes though the glucose of the salines does not. Effects of cysteine and a tissue extract are noteworthy.

RESULTS

Rate and stability of anaerobic glycolysis

Most of the present experiments concerned sliced guinea-pig cerebral cortex suspended in glucose–bicarbonate saline in equilibrium with N_2+CO_2. The extent of the formation of lactic acid, determined chemically in vessel contents at the end of experiments, and the extent of evolution of CO_2 from the bicarbonate, measured manometrically each 5 min., normally agreed (Table 2). Fig. 1 shows that the course of evolution of CO_2 is regular and reproducible until changed experimental conditions alter it.

Rates of glycolysis observed in different species and preparations are quoted in Table 3. Values with sliced cortex of rat and man are similar to those recorded by Dickens & Greville (1935), Weil-Malherbe (1938) and Elliott (1948), in their initial value and in their fall of some 20% in 90 min. Rates with guinea-pig tissues were similar. Table 3 shows that chopping the tissue to a suspension of particles 0·1–0·2 mg. in weight was not suitable for the present experiments as anaerobic glycolysis fell relatively rapidly, by 50–70% in 90 min.; fragments of 2–3 mg. in weight were therefore employed.

The cerebral cortex was not uniform in its level of anaerobic glycolysis (Tables 2 and 3). The outer slice, having the natural uncut outer surface of the hemisphere, yielded preparations of greatest glycolysis. Most of the remainder of the grey matter of the hemispheres was included in the next two slices cut, and these were 25% lower in glycolytic rate. With the agents studied, qualitative differences were not found between glycolysis in outer and inner slices. The difference in glycolytic rate may be compared with that in glycogen content (McIlwain & Tresize, 1956), and is not entirely explicable in terms of differences in swelling or dry weight of the tissues (see Experimental section).

Inhibition of anaerobic glycolysis

By electrical pulses. Applied electrical pulses under a variety of circumstances decreased glycolysis of both inner and outer slices (Table 4). Electrodes of gold, silver, and molybdenum were of comparable action, and under the conditions used no artifacts were induced by pulses in the absence of tissue. Agreement between manometric and chemical measures of glycolysis in individual

experiments was usually closer (see Table 2) than the standard deviations of Table 4 might imply; the values averaged in Table 4 are from experiments with different animals on different days, and were not always alike in the time at which pulses were applied.

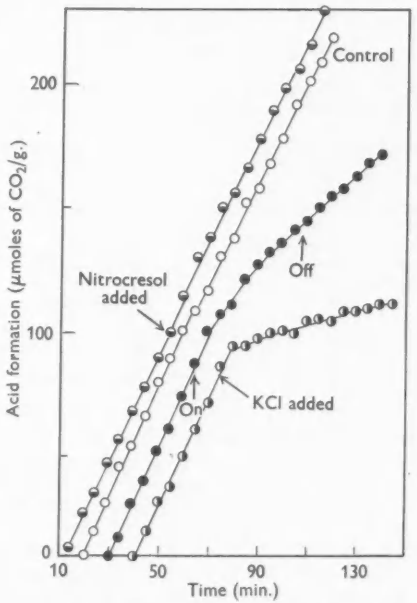

Fig. 1. Effect of pulses, 3:5-dinitro-*o*-cresol and potassium chloride on anaerobic glycolysis. Sliced guinea-pig cerebral cortex was in silver-grid electrodes *H* in bicarbonate–glucose saline. Condenser pulses of 15v peak potential and 0·3 msec. time constant were applied at 100/sec. for the time indicated between the arrows 'on' and 'off'. The nitrocresol in the same saline was added from a side arm at the time indicated by the arrow to give a 5×10^{-5}M solution; KCl was added similarly to give a 33 mM solution. All ordinates of some curves are displaced for clarity.

That the effect of pulses was directly on the tissue was further shown by applying them to grid electrodes in salines containing tissue slices, but with the slices floating outside the electrodes. Glycolysis was then unaffected, while that of tissues within electrodes in the same experiment was greatly decreased as shown in the last line of Table 4; mean rates of CO_2 evolution were: tissue in grid: initially, 125; during pulses, 100–60; after, 44; tissue outside grid: initially, 131; during pulses, 113–105; after, 100. This latter fall occurred also if no pulses were applied (Table 3).

Table 2. *Acid formation and lactate formation compared*

Experiments were of 90–120 min. Guinea-pig tissues were sliced and cut to fragments of mean wt. 2–3 mg. These (total wt. 60–70 mg.) were floated in 3·5 ml. of bicarbonate–saline in vessels *E* (Ayres & McIlwain, 1953) to which pulses (15–18v peak potential; time constant, 0·4 msec.) were applied as indicated. Lactic acid was determined according to Barker & Summerson (1941) and CO_2 from manometric observations. The CO_2 was measured directly for all except the first 10 and the last 1–5 min. of each experiment. During these periods it was assumed to be formed at the same rate as immediately after and before, and the total was then read by extrapolation from graphs such as Fig. 1. Observations on the same horizontal line refer to tissues from the same animal made in the same experiment.

	Vessels without pulses		Vessels with pulses	
Slice	Lactic acid (μmoles/g.)	CO_2 (μmoles/g.)	Lactic acid (μmoles/g.)	CO_2 (μmoles/g.)
Outer	273	255	137	128
	264	281	209	202
	269	258	263	239
	264	283	—	—
	302	281	—	—
Inner	171	178	145	129
	197	195	193	191
	227	225	162	155
	220	192	144	129
	183	185	140	127
	153	162	—	—

Table 3. *Rates of anaerobic glycolysis*

Rates were determined graphically from the course of CO_2 evolution from glucose–bicarbonate salines plotted as in Fig. 1. Fragments weighing 2–3 mg. were obtained by cutting slices 0·35 mm. thick, weighing them, and cutting with scissors. Chopped tissue included material from all depths in the cortex (mean wt. of fragments 0·1–0·2 mg.). Results are mean values of two or three experiments except when their number is stated in parentheses preceded by the S.E.M.

		Rate of evolution of CO_2 (μmoles/g./hr.) during		
Species	Preparation	0–30 min.	30–60 min.	60–90 min.
Man	Sliced and cut	106	81	81
Guinea pig	First slices, cut	161 ± 8·5 (14)	142 ± 5·5 (9)	134 ± 7 (9)
	Second slices, cut	121 ± 6·0 (12)	105 ± 6·6 (10)	99 ± 6·3 (10)
	Third slices, cut	119 ± 4·4 (11)	—	—
Guinea pig	Chopped	138	67	41
Rat	Chopped	122	66	45

Table 4. *Inhibition of glycolysis, using electrodes and pulses of various characteristics*

Inner slices or slice fragments of guinea-pig cerebral cortex (except*, in which chopped tissue was employed) were used in glucose–bicarbonate salines equilibrated with $N_2 + CO_2$ (95:5, v/v). Silver-grid electrodes *H* (Ayres & McIlwain, 1953) were used in vessels *A*; molybdenum and gold electrodes were in vessels *E*. When sufficient values are available the mean ±S.D. is quoted; others are averages of values differing by less than 15%. Anaerobiosis was maintained with yellow phosphorus except with gold electrodes, when the phosphorus was omitted. Alternating current is described by its frequency and virtual voltage.

Electrode type and material	Pulses	No. of expts.	Duration (min.) of expts.	Duration (min.) of pulses	Vessels without pulses: Lactic acid (μmoles/g.)	Vessels without pulses: CO_2 (μmoles/g.)	Vessels with pulses: Lactic acid (μmoles/g.)	Vessels with pulses: CO_2 (μmoles/g.)
Silver, grid	50 cyc./sec., 3 v	6	70	60	110	121	58±13	57±7
Silver, grid	2000 cyc./sec., 3·5 v	6	70	60	100	122	74±4	82±7
	Condenser pulses, time constant 0·4 msec. (v)							
Silver, grid	10	4	70	60	106	117	65±7	73±5
Gold, ring	15	5	95	30–35	200±21	195±16	153±13	142±17
Molybdenum, ring	18	3*	135	40–45	193	180	100	94
Molybdenum, ring	18	3	100	35	283	263	127	128
					Tissue outside electrodes, pulses applied			
Silver, grid	15	3	120	30	235	232	147	139

Table 5. *Glycolysis on applying pulses after different periods anaerobically*

Inner slices of guinea-pig cerebral cortex were suspended in vessels *E* and pulses of 18 v, 0·4 msec. applied from gold electrodes; the lower rates of glycolysis were established within 10 min.

Time of commencing pulses (min. after placing at 37°)	Rate before pulses (μmoles of CO_2/g./hr.)	Lower steady rate established by pulses (μmoles of CO_2/g./hr.)
10	—	40
35	118	46
65	123	43

Pulses required appreciable time to inhibit glycolysis, and the inhibition after it was established was not lost when pulses were stopped. Fig. 1 shows some 20 min. to be needed to lower a rate of 132 μmoles/g./hr. to 54 μmoles/g./hr. by pulses of a given intensity. Continuing the pulses for a further 20 min. did not further lower glycolysis, though other agents or pulses of greater intensity could do so. Thus the slightly greater effect of the pulses of Table 5 occurred within 10 min.

Pulses of given characteristics were of similar effect when applied at different periods during an experiment (Table 5).

Comparison with other agents. Pulses increase aerobic glycolysis of cerebral tissues and also their respiration, effects which are paralleled by several agents, including nitrophenols, phenazine salts and potassium salts (Dickens & Greville, 1935; Dickens, 1936; Weil-Malherbe, 1938; McIlwain, Anguiano & Cheshire, 1951; McIlwain, 1952). Some similarities and differences are seen in the actions of these substances under anaerobic conditions. The effect of KCl parallels that of applied pulses (Fig. 1); like the pulses, its action has been shown to be irreversible in the sense that it is not restored to normal when the tissue is transferred to salines with ordinary levels of potassium salts (Dixon, 1949). 3:5-Dinitro-*o*-cresol, on the other hand, has no effect anaerobically in concentrations fully adequate for a large increase in aerobic glycolysis (Figs. 1 and 2). The two effects of potassium chloride, aerobically and anaerobically, though opposite in sense are brought about by similar concentrations of potassium salts.

Comparison of effect of pulses under aerobic and anaerobic conditions

The two effects are brought about by pulses of similar intensities. Fig. 2 shows that, with condenser pulses of 100/sec. and time constant 0·3 msec., a peak voltage of 10 v raises aerobic glycolysis to about half its anaerobic level. At this intensity, anaerobic glycolysis is depressed by about 40%. At 18 v, both effects are intensified. Aerobically and anaerobically, the actions of sine-wave alternating current are both greater at 50 cyc./sec. than at 1000 cyc./sec.

It has also been found that when the glycolytic response of a tissue is damaged anaerobically, subsequent aerobic glycolysis is affected. This

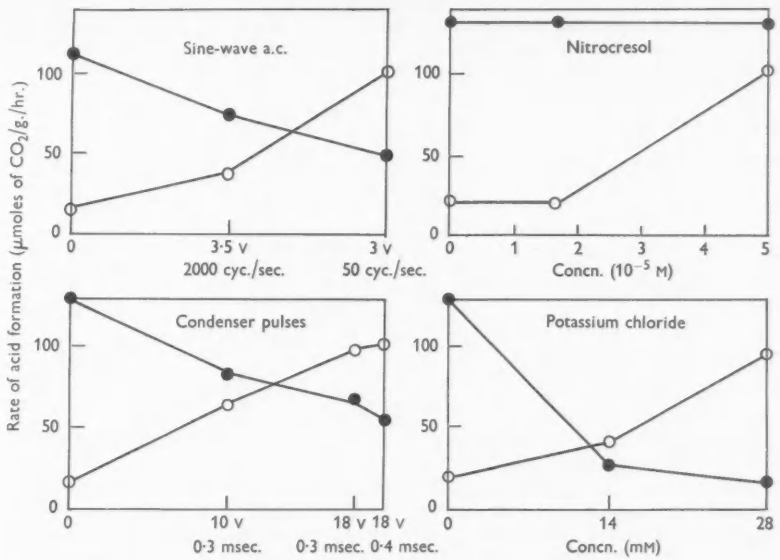

Fig. 2. Comparison of aerobic and anaerobic effects of pulses, KCl, and the dinitrocresol. Conditions for the anaerobic experiments (●) were as described in Fig. 1, except when otherwise indicated; aerobic experiments (○) employed the same tissue and apparatus, but were carried out in glucose–phosphate saline.

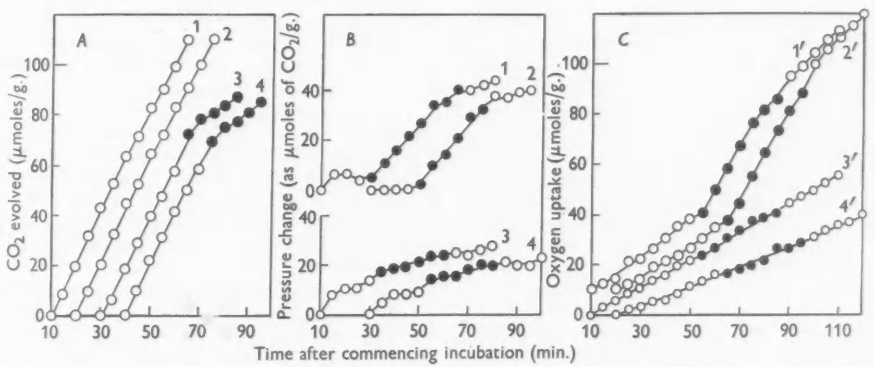

Fig. 3. Subsequent aerobic effects of pulses applied anaerobically. Part *A* was in vessels *E* with gold-ring electrodes; part *B* followed 10 min. after part *A*, the N_2+CO_2 of *A* being replaced by O_2+CO_2; conditions otherwise were the same. *C* gives the second, aerobic, part of an experiment in silver-grid electrodes *H* which commenced under the conditions of Expt. *A*, except that pulses were 15v and applied for 20 min., commencing 10 min. after the vessels were put at 37°. ○, No pulses applied; ●, pulses applied, in *A* and *B* at 18v peak potential, 0·4 msec. and in *C* at 15v, 0·4 msec., in all cases exponential and at 100/sec. Media: *A* and *B*, bicarbonate–glucose saline; *C*, phosphate–glucose saline. For manipulations, see text; and for lactate determined chemically, see Table 6. All ordinates of some curves are displaced for clarity.

Table 6. *Subsequent aerobic effects of pulses applied anaerobically*

Two sets of experiments are described here and in Fig. 3. The first, *A* and *B* of Fig. 3 and 1–6 of the table, lasted anaerobically for 65 min., and aerobically for 80 min. The second, *C*, of Fig. 3 and 1′–6′ of the table, lasted 36 min. anaerobically, and 110 min. aerobically. The order in which the vessels were handled in the experiments was 1, 3, 6, 5, 4, 2. Pulses (see Fig. 3) were applied during the aerobic part of the experiments.

Vessel (see Fig. 3)	Pulses during anaerobic part	Vessel removed immediately (see Fig. 3)	Lactic acid in vessel when removed (μmoles/g. of tissue)	Change in lactic acid aerobically (μmoles/g.)
1	–	After *B*	196	+37
2	–	After *B*	210	+51
3	+	After *B*	140	– 2
4	+	After *B*	152	+10
5	–	Before *B*	159	—
6	+	Before *B*	142	—
1′	–	After *C*	70	+70
2′	–	After *C*	77	+77
3′	+	After *C*	28	+28
4′	+	After *C*	39	+39
5′	–	Before *C*	103	—
6′	+	Before *C*	89	—

action of condenser pulses is shown in Fig. 3 and Table 6. Tissues whose anaerobic glycolysis had been decreased by pulses were of normal glycolytic rate aerobically until pulses were applied, when they proved incapable of response. The mean value for lactate formed during the aerobic part of the experiment was 4 μmoles/g. (Table 6) in distinction to the 44 μmoles/g. from the tissues not treated electrically in the anaerobic part. This effect of pulses is in contrast to their action under aerobic conditions when glycolysis can be increased repeatedly (McIlwain *et al.* 1951).

Pulses applied anaerobically affect also the subsequent respiratory response of the tissue to pulses. The experiment of Fig. 3*C* was commenced in the same way as that of the preceding paragraph, except that pulses were applied to vessels 3′ and 4′ earlier, and the anaerobic part of the experiment lasted only 36 min. The vessels were then removed from the manometers, their bicarbonate–saline was removed, the vessels and tissue (held by the grid electrodes) were washed twice with 2 ml. of oxygenated phosphate–saline at room temperature, and NaOH and paper were placed in the centre wells; oxygen was passed to equilibrate, and the vessels were returned to 37°, when the observations of Fig. 3*C* were made. The low initial respiratory rates in this figure (30–45 μmoles of O_2/g./hr.) would be expected to follow from the anaerobiosis and change of media (compare McIlwain, 1953). However, pulses yielded rates of 99–110 μmoles/g./hr. in those tissues which had not received pulses anaerobically, while those which had showed no subsequent respiratory response. In this case, as fresh media had been provided for the aerobic part of the experiment, the lactic acid formed then could be observed directly. In Table 6, glycolysis in the tissue previously exposed anaerobically to pulses was at normal average rates of 15 and 21 μmoles/g./hr. In the tissue not so exposed the level was markedly higher, presumably owing to the tissue's continuing ability to respond to pulses. By the end of the experiment of Fig. 3*C*, the tissues not exposed to pulses anaerobically had returned to normal respiratory rates of 60 μmoles/g./hr.; those exposed remained lower.

Attempted prevention of anaerobic effects of pulses

Many mechanisms can be envisaged for the anaerobic depletion, and clues to its nature may be obtained by attempting to restore or antagonize the change induced by pulses. Restoration has not been achieved, but some substances have been found to antagonize the pulses.

No antagonizing effect was shown by fumaric acid, the substance most effective in restoring respiratory response to aerobically depleted tissue (McIlwain & Gore, 1953). Tissue extracts gave complex effects, for many themselves lowered anaerobic glycolysis, probably because they contained glutamic acid or glutamine (see Weil-Malherbe, 1938). Nevertheless, some antagonism to pulses was suggested, and a number of substances likely to be present in such extracts were examined. In view of the antagonism found by Wollenberger (1955) between protoveratrine and nicotinamide acting on anaerobic glycolysis, nicotinamide was tested and found active (Table 7; this result was first obtained by Miss J. L. Lewis). Nicotinic acid had little effect, but adenine also antagonized. A nucleic acid and ethylenediaminetetraacetic acid were ineffective. Further studies are in progress; some concerning anticonvulsant agents and sine-wave alternating currents have

Table 7. *Antagonism to anaerobic depletion by pulses*

Cut slices of cerebral cortex were suspended in gold-ring electrode vessels *E* some of which contained the added substances in glucose–bicarbonate salines with N_2+CO_2. Condenser pulses at 100/sec. and of peak potential 15 v, time constant 0·4 msec., were applied after 30–40 min. for 30–40 min., and the course of glycolysis was measured for about 100 min. in all. The decreases are computed from the initial and final rates.

Addition	Decrease in rate of anaerobic glycolysis by pulses (μmoles/g. of tissue/hr.) Without addition	With addition
Sodium fumarate, 20 mM	90	89
Ethylenediaminetetraacetic acid, 1 mM	109	103
Yeast nucleic acid, 10 mg./ml.	110	104
Adenine, 30 mM	118	48
Nicotinamide, 30 mM	92	42

already been reported (Greengard & McIlwain, 1955), and show that relatively low concentrations of given agents can be effective and can differ in their ability to antagonize pulses of different electrical characteristics.

DISCUSSION

A working hypothesis linking changes described now and previously (McIlwain & Gore, 1953) is as follows. Pulses aerobically in presence of glucose affect some responsive system in the tissue akin to that involved in its normal physiological activity. This brings into play the normal increase in respiration associated with increased activity and presumably coupled to processes which restore the tissue to its previous state of responsiveness. If oxygen or glucose is not available, such restoration does not occur. Several systems, themselves complex, are involved between the pulse and its response; they may be summarized as (McIlwain & Gore, 1953); (1) substrate-level changes; (2) phosphate changes; (3) ion transport; (4) membrane polarization. Certain of these or of their components have presumably failed under the different conditions examined.

Previously induced aerobic depletion was partial. The respiratory response specifically failed, leaving the glycolytic response; glycolysis in absence of pulses remained below the level to which pulses could raise it. This, and a restoration of respiratory response by malate or fumarate, suggested the defect to be in (1) above. The anaerobic depletion observed in the present experiments involves both responses. Although a way of restoring the changes which are induced by the pulses has not been discovered, agents antagonizing the changes have been found and may give more information on processes (3) or (4); the respiratory defect is distinct from that induced previously in not being restored by fumarate. Further details of system (2) have been obtained by Heald (1956). By such studies it is hoped to find more chemical detail of the linkage between applied pulse and metabolic response.

SUMMARY

1. Anaerobic glycolysis proceeded in outer slices from the cerebral cortex of guinea pigs at about 160 μmoles/g./hr. and in inner ones at about 130 μmoles/g./hr. Each rate fell about 20% after 90 min.; the fall was more rapid in more finely cut tissue, and comparable rates were observed in tissue from the rat and from man.

2. Electrical pulses of types which excite the brain *in vivo* or stimulate respiration and glycolysis aerobically *in vitro* decreased anaerobic glycolysis.

3. Decreased anaerobic glycolysis persisted after pulses were stopped, and the tissue so treated was found to have lost also its ability to respond aerobically to pulses, both in respiration and in glycolysis.

4. Several substances and preparations were without action on the glycolytic effect of pulses anaerobically, but the presence of adenine or of nicotinamide could partly prevent their effect.

I am greatly indebted to Mr A. McNeil and Miss M. A. Tresize for assistance during these experiments.

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Biochemical Studies of Toxic Agents

9. THE METABOLIC CONVERSION OF INDENE INTO *cis*- AND *trans*-INDANE-1:2-DIOL*

By C. J. W. BROOKS and L. YOUNG
Department of Biochemistry, St Thomas's Hospital Medical School, London, S.E. 1

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The biological actions of indene have received little attention. Administration of the hydrocarbon to dogs with biliary fistulae was shown by Smith & Whipple (1930) to increase the flow of bile and also to produce marked toxic effects. The toxicity of indene was investigated by Cameron & Doniger (1939) in connexion with the presence of the compound in certain coal-tar-naphtha insecticides. They concluded that indene is not highly toxic. It was found, for example, that rats (180–200 g. body wt.) survived the subcutaneous injection of 0·5 g. of the compound. Rats developed fatty livers and died, however, after doses of 1·0 g. of indene. Indene was administered to rabbits by Böhm (1941), who found that a dose of 1·0 g. was tolerated when it was given by mouth to animals weighing 2–3 kg. Böhm reported that administration of the hydrocarbon was followed by an increased excretion of ethereal sulphate and glucuronide, an observation which suggests that indene undergoes hydroxylation in the animal body.

Studies of the metabolism of aromatic hydrocarbons (e.g. Boyland & Levi, 1935; Young, 1947; Boyland & Wolf, 1948; Booth & Boyland, 1949; Corner & Young, 1954, 1955) have shown that naphthalene, anthracene and phenanthrene give rise to dihydrodiols in addition to phenolic derivatives. These metabolites appear in the urine in the free state and in conjugated form. Indene is of interest because dihydrodiol formation might occur in the aromatic ring or at the double bond in the 5-membered ring. In the present work it has been shown that in the rabbit and the rat indene gives rise to indane-1:2-diol (1:2-dihydroxyindane) in which the aromatic ring is intact.

EXPERIMENTAL

Only male animals were used. Rabbits (2–3 kg. body wt.) were fed on cabbage together with rat cakes [J. Murray and Sons (London) Ltd.]. Rats were fed on rat cakes only. The animals had access to water at all times. They were housed in metabolism cages which permitted the collection of urine separate from faeces. Urine was collected daily and stored in the refrigerator.

Indene was purified by distillation and the fraction of b.p. 177–179° was used for dosing within 2 hr. of its collection. When rabbits received indene by stomach tube the dose was suspended in 10 ml. of water and washed down with a further 5–10 ml. of water. Undiluted indene was used for subcutaneous injection into rabbits and for administration to rats by stomach tube.

Reference compounds

Indene bromohydrin was prepared according to Suter & Milne (1940) and, when recrystallized from $CHCl_3$–light petroleum, formed needles, m.p. 129–130°. The direct conversion of indene bromohydrin into *trans*-indane-1:2-diol, as described by Porter & Suter (1935), gave, in our hands, only poor yields of diol. Satisfactory yields were obtained, however, from indene epoxide.

Indene epoxide was prepared by a modification of the procedure of Whitmore & Gebhart (1942) in which the ether-extraction and evaporation described by these authors were avoided in view of the ready volatility of the epoxide. Indene bromohydrin (25·9 g.), m.p. 125–127°, dissolved in a mixture of dioxan (60 ml.), ethanol (50 ml.) and water (15 ml.) was treated with 65 ml. of 1·91 N-KOH (1·02 mol. prop.) added during 25 min. with continuous shaking. The solution was allowed to stand for 5 min., and was then poured into ice water (500 ml.). The resultant oily precipitate crystallized on rubbing. The mixture was left overnight at 5°, and the solid was filtered off and washed with water. The product consisted of 14 g. of indene epoxide (87% yield), m.p. 31–32°. This substance readily sublimed under reduced pressure at a bath temp. of 50° to give prisms, m.p. 31–32°. It gave no precipitate with 2:4-dinitrophenylhydrazine in 96% ethanol containing 4% (v/v) H_2SO_4, under conditions suitable for the rapid formation of a precipitate from indan-2-one. The ultraviolet absorption curve of indene epoxide in 95% ethanol showed: $\lambda_{max.}$ 210, 259, 266, 272·5 and 274 mμ.; log ε, 3·78, 2·64, 2·69, 2·58 and 2·61 respectively.

Indene epoxide was converted into a mixture of *cis*- and *trans*-indane-1:2-diol by treatment with 1% (v/v) acetic acid on the steam bath (Loon, 1919). The *trans*-diol was separated from the mixture by virtue of its sparing solubility in $CHCl_3$, the $CHCl_3$ filtrate was evaporated to dryness *in vacuo*, and the *cis*-diol was obtained on recrystallization of the residue from *cyclo*hexane. In agreement with Loon, the principal product, obtained in 50% yield, was the *cis*-diol (m.p. 98–99°); the yield of *trans*-diol (m.p. 158–159°) was 13%. The yield of hydration products is probably lowered by the formation of indan-2-one. In an experiment in which indene epoxide was suspended in water and steam-distilled, indan-2-one was isolated from the distillate in 20% yield (as its 2:4-dinitrophenylhydrazone) (cf. Loon, 1919).

cis- and *trans*-Indane-1:2-diol were further characterized as their dibenzoates (Loon, 1919), prepared in the usual way with benzoyl chloride in pyridine at room temp. The

* Part 8: Corner & Young (1955).

cis-dibenzoate was separated by ether extraction, purified by recrystallization from ether and obtained as needles, m.p. 110–111°. The *trans*-dibenzoate could not be crystallized from the crude product, which was therefore chromatographed on alumina. The fraction eluted by light petroleum (b.p. 60–80°)–benzene (99:1) when recrystallized from methanol gave the dibenzoate, m.p. 77–78° (yield, 52%).

Indan-2-one was prepared from *trans*-indane-1:2-diol. The diol (580 mg.) dissolved in 2N-HCl (40 ml.) was heated on the steam bath under a reflux condenser for 1 hr. The reaction mixture, including the crystalline deposit in the condenser, was extracted with ether, giving a crude product from which indan-2-one (428 mg.), m.p. 54–56°, was isolated by chromatography on alumina. No other crystalline product was detected. Indan-2-one was also prepared from *cis*- and *trans*-indane-1:2-diol by refluxing in 7% (v/v) H_2SO_4 (Porter & Suter, 1935). The ketone, purified by steam distillation, had m.p. 58–60°: when kept at room temp. it decomposed with the formation of a yellow gum, but it was stable at 5° if stored in the dark. Indan-2-one was characterized as the oxime, which formed plates from $CHCl_3$–light petroleum (b.p. 40–60°), m.p. 151–152° (Porter & Suter, 1935), and as the 2:4-dinitrophenylhydrazone obtained from $CHCl_3$–ethanol as orange-yellow needles, m.p. 204–205° (decomp.) (Suter & Milne, 1940).

Paper-chromatographic procedure

The solvent mixtures and conditions used are given in Table 1.

Two methods of detecting 1:2-glycols on paper chromatograms were described by Buchanan, Dekker & Long (1950), and have been applied to the indane-1:2-diols. (1) The paper was dried and sprayed lightly with xylene, followed by lead tetraacetate in benzene (1%, w/v). It was advantageous to steam the paper at this stage to hydrolyse the reagent and reveal at once the white spots against a brown background. Although the reagent was not stable on storage, this disadvantage was overcome by using acetic acid as the solvent instead of benzene (Bush, 1955). However, the method was not very satisfactory for our purpose as 50 μg. of indane-1:2-diol was required to give a well-defined spot after development of the chromatogram.

Table 1. *Chromatographic separation of* cis- *and* trans-*indane*-1:2-*diol*

Whatman no. 1 paper and the descending method were used. Time of run: 16–18 hr. at room temp. Solvent mixtures (all proportions by vol.) were shaken vigorously for 1–2 min. and allowed to separate for 30 min. The upper layer was used as the mobile phase and the lower layer as the stationary phase. For methods of detection of spots see text.

Solvent system	R_F values: *cis*	R_F values: *trans*
1. Benzene–water–ammonia (sp.gr. 0·88) (5:4:1)	0·28	0·05
2. Benzene–water–acetic acid (5:4:1)	0·36	0·06
3. Benzene–water–ethanol (5:4:1)	0·38	0·12
4. Benzene–water–ethyl acetate–acetic acid (4:4:1:1)	0·60	0·38
5. Water	0·70	0·69

(2) The dried chromatogram was sprayed with 2% (w/v) aqueous $NaIO_4$, left for 10–15 min. in the air, immersed in SO_2 vapour until the iodate and periodate were fully reduced to iodide, and finally sprayed with Schiff's reagent. Yellow spots slowly became visible against a background which changed from white to greyish-mauve. Even though colour development could be hastened by steaming the paper, it remained very slow, especially with the *trans*-diol. It was eventually found that excellent results were obtained by omitting the SO_2 treatment, when the yellow spots appeared as soon as the Schiff's reagent was applied. Under these conditions the final background colour was magenta.

In Table 1 are summarized R_F values for *cis*- and *trans*-indane-1:2-diol in five solvent systems.

Isolation of metabolites from the urine of rabbits dosed with indene by stomach tube

In one experiment with four rabbits the urine was collected daily for three successive 3-day periods. At the beginning of the second 3-day period the animals were dosed by stomach tube with indene (0·2 g./kg. body wt.) and a similar dose was given 1 day later. The total amount of indene administered was 3·7 g. The three 3-day batches of urine were examined separately by the following procedure.

Extraction of the urine at pH 7·5. The urine was filtered through glass wool and about 200 ml. of each 3-day batch of urine was reserved for treatment with acid (see below). The remainder of each batch (1–1·5 l.) was adjusted to pH 7·5 and extracted continuously with ether for 20 hr. The ether extract was washed with M-$NaHCO_3$ and with 2N-NaOH. Each washing was shaken twice with ether and the ethereal extracts were combined with the main extract and evaporated to dryness under reduced pressure. The urine extracts for days 1–3 and for days 7–9 yielded only about 20 mg. of oily residue, whereas that for days 4–6 yielded 280 mg. of partly crystalline material. This was chromatographed on 9 g. of alumina (Savory and Moore Ltd.) and yielded two well-separated crystalline fractions: (i) 22 mg. eluted by benzene–ether (9:1), and (ii) 87 mg. eluted by ether–methanol (95:5). Fraction (ii), when recrystallized from $CHCl_3$, gave prisms (46 mg.) of compound *A*, m.p. 157–159°, $[\alpha]_D$ +5° in ethanol (*c*, 1·02). Fraction (i) on recrystallization from ether–light petroleum (b.p. 40–60°) gave fine needles (10 mg.) of compound *B*, m.p. 109–111°, $[\alpha]_D$ +43° in $CHCl_3$ (*c*, 0·74).

Chromatographic separation, though convenient, was not essential, for compound *B* could be extracted from the crude mixture with boiling *cyclo*hexane or light petroleum, and compound *A* was obtainable by crystallization of the residue from $CHCl_3$. The characterization of compounds *A* and *B* is described below.

Extraction of the urine at pH 2. After the extraction at pH 7·5, each batch of urine was acidified with conc. HCl to pH 2 and a 500 ml. portion was extracted with ether for 20 hr. The ether extract was washed with M-$NaHCO_3$, N-NaOH and water. Each washing was shaken twice with an equal volume of ether, and these ether extracts were combined with the main ether extract and evaporated to dryness under reduced pressure. The extract from the urine of days 1–3 yielded only a trace of oil; that for days 4–6 gave 149 mg. of oily residue. Chromatography on alumina (6 g.) resulted in the separation of two sharply defined fractions of mainly crystalline character: (i) 13 mg.

eluted by benzene–ether (4:1) which on recrystallization from ether–*cyclo*hexane gave compound *B* (2 mg.), m.p. 105–107°, and (ii) 80 mg. eluted by ether–methanol (95:5) which on recrystallization from $CHCl_3$ gave compound *A* (28 mg.), m.p. 156–157°.

The ether-soluble acids in the $NaHCO_3$ extracts were examined by paper chromatography but no metabolites of indene were detected.

Isolation of metabolites from the urine of rabbits dosed subcutaneously with indene

Undiluted indene (1 g./kg. body wt.) was injected under the skin of the backs of three rabbits. After 2 days, the animals again received the same dose of the hydrocarbon. Urine was collected for 5 days after the first dosing. One rabbit died on the fifth day. The survivors eventually developed lesions at the sites of injection. The total amount of indene given was 15·3 g. The urine was extracted continuously with ether at pH 7·5 as in the experiments already described. The total crude residue from the combined extracts amounted to 440 mg., from which were separated 84 mg. of phenolic material and 265 mg. of neutral material. The latter was chromatographed on alumina (8 g.), affording compound *B* (139 mg. of crude fraction, yielding 96 mg. of product, m.p. 96–104°) and compound *A* (33 mg. of crude fraction, yielding 20 mg. of product, m.p. 155–158°).

Identification of metabolites isolated from the urine of rabbits dosed with indene

Various experiments were carried out in which the dose of indene administered by stomach tube ranged from 0·2 to 1·0 g./kg. body wt., and these led regularly to the isolation of compounds *A* and *B*. The identification of these metabolites was complicated by their occurrence as mixtures of optical isomers which tended to melt indefinitely. They were characterized as *trans*- and *cis*-indane-1:2-diol, however, by the following observations.

Compound A. Different samples of compound *A* had m.p.'s between 155° and 160°, and occasionally the melt remained cloudy until 175–180°: there was no depression in m.p. on admixture with (±)-*trans*-indane-1:2-diol of m.p. 158–159°. (Found: C, 71·6; H, 6·8. Calc. for $C_9H_{10}O_2$: C, 72·0; H, 6·7%.) $[\alpha]_D$ in ethanol varied between −7° and +5°. The compound reacted slowly with lead tetraacetate in acetic acid (1·15 mol. prop. of oxidant consumed in 20 hr.) and with periodic acid in aqueous ethanol (1:1) (0·95 mol. prop. of oxidant consumed in 25 hr.). Treatment of compound *A* (24 mg.) with 2N-HCl (5 ml.) on the steam bath for 40 min. yielded a crystalline product (17 mg., m.p. 58–60°) which was isolated by steam distillation and identified as indan-2-one by m.p. and mixed m.p. with an authentic sample (m.p. 58–60°). The derived 2:4-dinitrophenylhydrazone had m.p. 204–205° (decomp.) and gave no depression on admixture with an authentic specimen of the same m.p.

Compound B. The m.p. of compound *B* varied within the range 94–111°: the higher m.p.'s seemed to be associated with a higher degree of optical purity. Thus a sample of m.p. 94–96° had $[\alpha]_D$ +31° in $CHCl_3$ (*c*, 1·15), whereas one of m.p. 109–111° had $[\alpha]_D$ +43° in $CHCl_3$ (*c*, 0·74). (Found: C, 71·6; H, 6·5. Calc. for $C_9H_{10}O_2$: C, 72·0; H, 6·7%.) On admixture of various samples of compound *B* with (±)-*cis*-indane-1:2-diol of m.p. 98–99°, the m.p. was in no case depressed below that of the lower-melting component. Compound *B* reacted very rapidly with lead tetraacetate in acetic acid (1·03 mol. prop. consumed in 10 min.) and with periodic acid in aqueous ethanol (1:1) (0·95 mol. prop. consumed in 20 min.). Treatment of compound *B* (60 mg.) with benzoyl chloride (0·25 ml.) in pyridine (1 ml.) at room temp. overnight yielded an oily product which was purified by chromatography on alumina. A mixture of light petroleum (b.p. 60–80°) and benzene (90:10) eluted a crude crystalline fraction (95 mg., m.p. 89–92°) which was recrystallized from ether–light petroleum as prisms, m.p. 91–92°, $[\alpha]_D$ −32° in $CHCl_3$ (*c*, 1·26). (Found: C, 76·9; H, 5·2. Calc. for $C_{23}H_{18}O_4$: C, 77·1; H, 5·1%.) Treatment of compound *B* with 2N-HCl at 100° for 1 hr. resulted in the formation of indan-2-one, which was identified by comparison with an authentic sample.

In confirmation of these results, comparison of the ultraviolet absorption spectra of compounds *A* and *B*, and of the synthetic *trans*- and *cis*-indane-1:2-diol, measured in 95% (v/v) ethanol, showed them all to be identical over the range 210–300 mμ., with maxima at 211, 259, 265 and 271·5 mμ., log ε 3·94, 2·76, 2·92 and 2·94 respectively.

The rates of reaction of the diols with lead tetraacetate in acetic acid were also determined. The reactions were conducted at 21·5° with equimolar concentrations of diol and reagent, and the rate constants were derived by plotting the time of reaction against the inverse concentration of diol. The extremely rapid reaction of the *cis*-diol necessitated the use of low concentrations (0·00013M) of reactants: even so the time of half-reaction was only 15 sec. The course of the reaction was followed by titration with 0·002N-$Na_2S_2O_3$. Compound *B* and the synthetic (±)-*cis*-diol reacted at the same rate, $k_{21·5}$ = 32 000 l./mole/min. Compound *A* and the synthetic (±)-*trans*-diol (0·013M) also reacted at the same rate, $k_{21·5}$ = 0·52 l./mole/min. Criegee, Kraft & Rank (1933) recorded for k_{20} values of 27 800 and 0·467, respectively, for *cis*- and *trans*-indane-1:2-diol.

Products formed by acid treatment of the urine of rabbits dosed with indene

These experiments were carried out with the urine of rabbits dosed with indene by stomach tube (see above). The following treatment was applied to measured portions (approx. 200 ml. each) of (1) the urine of days 4–6, (2) the same urine after extraction with ether at pH 7·5 and at pH 2, as already described, and (3) the urine of days 7–9.

The urine was diluted with water (100 ml.) and steam-distilled, and 80–100 ml. of distillate (*a*) was collected. The urine was cooled and treated with sufficient 50% (v/v) H_2SO_4 to give a concentration of 7–8% (v/v) H_2SO_4, then refluxed gently for 1·5 hr. Any crystalline deposit forming in the condenser at this stage was dissolved in ether and combined with (*b*). The residual liquor was then steam-distilled and 150 ml. of distillate (*b*) collected. Each of the distillates was extracted four times with 20 ml. portions of ether, and the combined extracts were evaporated *in vacuo* at room temp., and finally at 0°, to 10–15 ml. The concentrate was decanted from ice, diluted with ethanol (10 ml.) and treated with a solution of 2:4-dinitrophenylhydrazine (12 ml., 20 mg./ml.) in 96% (v/v) ethanol containing 4% (v/v) H_2SO_4.

Precipitates were obtained only from the distillates (*b*) and only from the urine of days 4–6. These precipitates were identified as indan-2-one 2:4-dinitrophenylhydrazone by m.p. and mixed m.p. with an authentic specimen. The combined material was recrystallized from $CHCl_3$–ethanol and the product had m.p. 205–206° (decomp.) (Found: C, 57·7; H, 4·1. Calc. for $C_{15}H_{12}O_4N_4$: C, 57·7; H, 4·0.) Almost identical yields of phenylhydrazone were obtained from the ether-extracted and unextracted urine. The total amount isolated corresponded to approximately 25% of the dose of indene.

Isolation of metabolites from the urine of rats dosed with indene by stomach tube

Twenty-four rats (body weight 200–250 g.) were dosed with indene by stomach tube. Each rat received a single dose of 0·25 g. and urine was collected daily for 3 days after dosing.

The urine was extracted with ether (at pH 7·5) as described above. The combined extracts gave on evaporation a partly crystalline amber-coloured residue (630 mg.), which was extracted with 50 ml. of boiling light petroleum (b.p. 60–80°). The petrol-insoluble residue (380 mg.) was extracted three times with boiling *cyclo*hexane (50 ml. portions), and 210 mg. remained undissolved. The *cyclo*hexane extract, on cooling, deposited fluffy crystals, which when recrystallized from $CHCl_3$ gave 22 mg. of colourless prisms, m.p. 184–186°, $[\alpha]_D$ +31° in $CHCl_3$ (*c*, 0·75). The nature of this substance (compound *C*) is considered below. The petrol-soluble material, recrystallized from ether–light petroleum, gave compound *B* (32 mg.), m.p. 109–111° (alone and mixed with compound *B* from rabbit urine), $[\alpha]_D$ +44° in $CHCl_3$ (*c*, 1·01).

After removal of the crystalline products, the remaining material was recombined, and a neutral fraction (240 mg.) was separated and chromatographed on alumina (7 g.). This afforded a further 98 mg. of compound *B*, m.p. 108–111°. Although some solid was eluted at the point where compound *A* would have been expected to appear, no crystalline product could be isolated.

A portion of the extracted urine was treated with sulphuric acid as described above for the isolation of products formed by acid treatment of rabbit urine. Indan-2-one 2:4-dinitrophenylhydrazone was isolated in a yield representing 5% of the administered indene.

Nature of compound C *obtained from the urine of rats dosed with indene*

The substance of m.p. 184–186° showed no change in m.p. or appearance on recrystallization successively from $CHCl_3$ and acetone–light petroleum. Amounts of 20 μg. on paper could readily be detected by the periodate–Schiff's reagent method (see below). The resulting yellow spots closely resembled in character and in time of development those observed with (±)-*trans*-indane-1:2-diol. Paper chromatograms of compound *C* developed with solvent mixture 4 (Table 1) gave somewhat elongated spots with R_F value (0·34) slightly less than that (0·39) found for (±)-*trans*-indane-1:2-diol run simultaneously.

To test the homogeneity of compound *C* it was dissolved in benzene and chromatographed on alumina (1 g.). Elution with ether (350 ml.) gave 14 mg. of crystalline material, m.p. 184–186°; ether–methanol (9:1) eluted a further 5 mg. of product with the same m.p. These fractions, when separately recrystallized from $CHCl_3$, both yielded crystals of m.p. 185–186°. The two samples gave no depression in m.p. on admixture. The two fractions were combined, and a sample was taken for measurement of the rate of reaction with lead tetraacetate in acetic acid. From the time of half-reaction, the rate constant ($k_{21\cdot5}$) was found to be 0·53 l./mole/min. The ultraviolet light absorption of compound *C* in 95% ethanol showed maxima at 211, 259, 265·5 and 272 mμ., log ϵ 3·92, 2·73, 2·92 and 2·95 respectively, and was not significantly different from that of (±)-*trans*-indane-1:2-diol over the range 210–300 mμ.

While the properties of compound *C* suggest that it may be (+)-*trans*-indane-1:2-diol, further evidence is required to confirm this.

Observations on the interconversion of cis- *and* trans-*indane*-1:2-*diol*

The interconversion of the diols was studied by isolating the products formed from each diol under various conditions, and by paper-chromatographic methods.

Effect of acid and base on the diols. *cis-* and *trans-*Indane-1:2-diol were each recovered in 90% yield after refluxing for 2 hr. in N-NaOH (in aqueous ethanol, 50%, v/v). Treatment of the *cis*-diol (200 mg.) with 0·02N-H_2SO_4 on the steam bath for 45 min. gave a crude product (198 mg., m.p. 90–96°) from which *trans*-diol (15 mg., m.p. 153–156°) was isolated by recrystallization from $CHCl_3$. In a similar experiment, Hermans (1924) obtained a 7% yield of *trans*-diol.

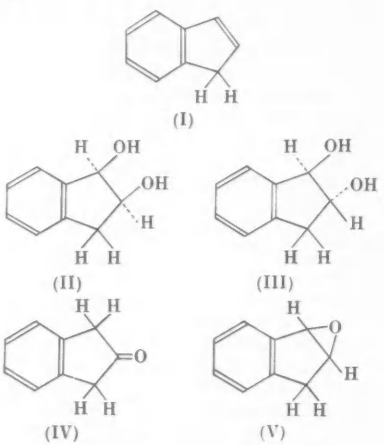
The interconversion of the diols was studied by heating *cis-* or *trans*-diol (200 mg.) on the steam bath in 20 ml. of 0·2N-H_2SO_4 for 5 hr. The solution was then extracted continuously with ether for 2 hr. and the extract was evaporated to dryness *in vacuo* at room temp. The residue, which in each case exceeded 185 mg., was recrystallized from $CHCl_3$ to yield the *trans*-diol, and the mother liquor from this was evaporated to dryness to give crude *cis*-diol. This was purified by recrystallization from *cyclo*hexane. The identity of each of these products was established by m.p. and mixed m.p. Under these conditions the *cis*-diol gave rise to 57% *trans-* and 28% *cis*-diol, and the *trans*-diol afforded 59% *trans-* and 27% *cis*-diol. In the experiment starting with *cis*-diol, indan-2-one was also isolated in a yield of 2·5% as its 2:4-dinitrophenylhydrazone.

Stability of the diols in rabbit urine at different pH *values.* In these experiments a sample of normal urine obtained from a pair of rabbits, under the same conditions as in the metabolism experiments, was adjusted to the required pH with 2N-HCl or 2N-NaOH. Samples of the indane-1:2-diols (10 mg.) were dissolved in 20 ml. portions of the urine, and the solutions were left at room temperature in flasks open to the air, the pH being readjusted if

necessary. One day later the pH was brought to 7·5 and each solution was extracted continuously with ether for 3 hr. The extracts were evaporated to dryness *in vacuo*, the residues (8–10 mg.) were each dissolved in 1 ml. of acetone, and 0·10 ml. portions were applied to paper chromatograms. These were developed with solvent mixture 4 (Table 1). The spots obtained were compared with those found in parallel runs in which mixtures of the diols (95:5, w/w) were added to urine and treated in the same way. Experiments were performed with both *cis*- and *trans*-diol in urine at pH 5, 6·5 and 8–9. In no case was any isomerization detected.

DISCUSSION

Those aromatic hydrocarbons which are converted into dihydroaromatic diols in the animal body undergo hydroxylation at their more reactive bonds, and this suggested that in indene (I) the double bond in the 5-membered ring would be a site of biochemical oxidation. This has been shown to be so, for *cis*- and *trans*-indane-1:2-diol (II and III) were found in the urine of rabbits after administration of indene by stomach tube or by subcutaneous injection.



The complete characterization of the isolated diols was rendered difficult by their occurrence as mixtures of optical isomers. The degree of optical homogeneity could not be estimated, since the two indane-1:2-diols do not appear to have been resolved into their enantiomorphic pairs. Contrary to the reports of Dox (1923) and Ingersoll (1944) no resolution of the (−)-menthylurethane of *trans*-indane-1:2-diol was recorded by Loon (1919). In the case of the *cis*-diol, the virtual identity of the specific rotations (43° and 44°) of the metabolites excreted by the rabbit and the rat suggests that these may approximate to the (+)-form. Apart from the question of their optical purity, the diols were identified as *cis*- and *trans*-indane-1:2-diol principally on the following evidence. (1) Both diols were converted by hot dilute acid into indan-2-one, characterized as its 2:4-dinitrophenylhydrazone. (2) The two diols, as well as authentic (±)-*trans*- and (±)-*cis*-indane-1:2-diol, all showed the same light-absorption, measured in 95% ethanol, over the range 210–300 mμ. (3) The isolated diols each consumed one molar equivalent of periodic acid, one reacting very slowly and the other very rapidly. Lead tetraacetate gave similar results, and the rate constants for the reactions in acetic acid were identical with those observed with (±)-*trans*- and (±)-*cis*-indane-1:2-diol under the same conditions.

From the small amounts of the diols isolated it appeared likely that other metabolites of indene were being excreted. The urine of the rabbits dosed by stomach tube contained material, not extracted by ether, which afforded indan-2-one (IV) when the urine was refluxed with H_2SO_4 (7%, v/v). The ketone was isolated in amounts representing about 25% of the indene administered. A simple explanation of this finding is that the ketone arises from conjugates of the diols with glucuronic acid or sulphuric acid. This view receives support from the increased excretion of 'glucuronide' which was observed with two pairs of rabbits after dosing them with indene (0·5 g./kg. body weight); the increments corresponded, on an equimolar basis, to 30 and 39% of the indene administered.

The formation of both *cis*- and *trans*-diols is noteworthy since the diols isolated as metabolites of aromatic hydrocarbons have been assigned the *trans*-configuration (Boyland, 1950; Cook, 1950). It appears unlikely that interconversion of *cis*- and *trans*-indane-1:2-diol occurs in the urine, for samples of each diol added to urine at pH 5, 6·5 and 8–9 underwent no detectable isomerization in 24 hr. The interconversion of these diols is normally achieved only at low pH (Hermans, 1924; Suter & Milne, 1940), and in the present work it was shown that both diols are converted by 0·2N-H_2SO_4 at 95–100° into an equilibrium mixture of *cis*- and *trans*-diol in the approximate ratio of 1:2. Although the direct isomerization of the diols in the urine appears unlikely, the possibility remains that the two diols isolated might have arisen from the breakdown in the urine of a derivative of one of them. Hydrolysis of a sulphate of one of the diols, for example, might be accompanied by some inversion of a hydroxyl group.

Boyland (1950) has drawn attention to the possible role of epoxides in the metabolic oxidation of aromatic hydrocarbons. Indene epoxide (V) undergoes cleavage by water to yield a mixture of

cis- and *trans*-diol, together with indan-2-one (Loon, 1919). So far, however, there is no experimental evidence to show that epoxide formation occurs in the animal body during the metabolism of indene.

SUMMARY

1. Indene has been administered to rabbits by stomach tube, and fractionation of ether extracts of the urine has yielded optically active *cis*- and *trans*-indane-1:2-diol in a total amount corresponding to about 5% of the dose. Acid treatment of the urine, either before or after ether extraction, yielded indan-2-one in amounts corresponding to about 25% of the dose of indene.

2. The same two diols have been isolated from the urine of rabbits dosed with indene by subcutaneous injection.

3. *cis*-Indane-1:2-diol has been isolated from ether extracts of the urine of rats dosed with indene by stomach tube. Indan-2-one has been obtained by acid treatment of the extracted urine.

4. The interconversion of *cis*- and *trans*-indane-1:2-diol has been studied, and the metabolic formation of the two diols is discussed.

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Studies of Sebum

6. THE DETERMINATION OF THE COMPONENT FATTY ACIDS OF HUMAN FOREARM SEBUM BY GAS–LIQUID CHROMATOGRAPHY*

By A. T. JAMES
National Institute for Medical Research, Mill Hill, London, N.W. 7

and V. R. WHEATLEY
Departments of Biochemistry and Dermatology, Medical College of St Bartholomew's Hospital, London, E.C. 1

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Relatively little information is available concerning the component fatty acids of human sebum. Engman & Kooyman (1934) made a brief study, using lead-salt separation and fractionation of the bromine derivatives of the liquid acids. They concluded that the free and combined fatty acid fractions of sebum contained both oleic and linoleic acids and that the combined acid fraction also contained arachidonic acid. They also demonstrated appreciable amounts of saturated products in the liquid acid fraction and concluded that branched-chain acids were possibly present. Ricketts, Squire & Topley (1951) examined the free fatty acids of human forearm sebum and were able to isolate pure barium oleate (identified by X-ray diffraction). They also reported a chromatographic examination of the saturated fatty acids performed for them by Dr G. A. Howard. This showed the

* Part 5: Wheatley (1954).

presence of at least seven components, of which the most abundant was probably palmitic acid.

Weitkamp, Smiljanic & Rothman (1947) made a comprehensive study of the free fatty acids of human-hair lipid. They fractionated by amplified distillation the free fatty acids obtained from the lipids from 45 kg. of hair. The source of hair was barber's shop sweepings, and contamination of sebum was likely; nevertheless, they were able to show that the extracted fatty acids constituted a homologous series from C_7 to C_{22}, which included both odd and even members. Both saturated and unsaturated acids were present, and the position of the double bonds in the latter was atypical since octadec-6-, -8- and -9-enoic acids were present, whereas the C_{16} unsaturated acid was hexadec-6-enoic acid and not palmitoleic acid. This unusual character of the acids was thought to preclude the possibility of extensive contamination of the hair lipids; nevertheless, it was essential that this work should be repeated on specimens of sebum obtained as free as possible from contamination. Furthermore, it has been observed by Burtenshaw (1942) and by Ricketts *et al.* (1951) that the self-sterilizing power of human sebum appears to depend on the long-chain unsaturated fatty acids; Rothman, Smiljanic, Shapiro & Weitkamp (1947) showed that sebum possessed fungicidal properties associated with the shorter-chain saturated acids. It was, therefore, necessary to be able to determine the component fatty acids in a sample of sebum from a single subject in order to make possible the study of the role played by these acids in maintaining the health of the skin.

In view of the complex nature of the fatty acids of human sebum indicated by the work of Weitkamp *et al.* (1947) the method of choice must have a very high resolving power. Furthermore, sebum from certain animals, e.g. the sheep, contains a high proportion of branched-chain fatty acids, and it is therefore necessary for the method to be able to distinguish between straight- and branched-chain and saturated and unsaturated acids of a homologous series, each member differing by only one carbon atom. Conventional partition-chromatographic methods applied to the separation of fatty acids will usually separate acids which differ in chain length by two carbon atoms, but are useless if applied to sebum analysis. The method of gas–liquid chromatography developed by James & Martin (1952) for shorter-chain fatty acids has been extended (James & Martin, 1956*a*) to the methyl esters of higher fatty acids, and possesses the necessary high resolving power. When applied to sebum fatty acids it proved entirely suitable, and since less than 10 mg. of material is required for an analysis it can be applied to individual sebum samples.

EXPERIMENTAL

Sebum. A sample of sebum was collected from the forearms of six normal male subjects by the acetone method and was separated into free and combined fatty acids and non-saponifiable material by methods already described (MacKenna, Wheatley & Wormall, 1950). The sample contained: free fatty acids 26·3%, combined fatty acids 35·5% and unsaponifiable matter 33·8%. The fatty acid fractions were esterified with methanol and H_2SO_4 and analysed on the gas–liquid chromatogram.

Gas–liquid partition chromatography. The columns used had as stationary phase the high-boiling lubricating oil described previously (James & Martin, 1956*a*). Samples were applied to the column with a micropipette (James & Martin, 1956*b*). Column temperatures were maintained at 197° with boiling ethylene glycol. Bromination of the mixed esters was carried out as described by James & Martin (1956*a*).

The measurement of peak areas was carried out by drawing lines through the points of inflexion in the sides of each peak. The area enclosed by the triangle so formed is about 4% less than the area of the peak provided that the latter approximates closely to the shape of the Gaussian error curve (this is usually the case).

The response of the gas-density balance (Martin & James, 1956) is proportional to the mol.wt. excess of the substance being detected (i.e. mol.wt. – mol.wt. of nitrogen, the carrier gas). The peak areas were corrected to those expected for a standard substance, in this case methyl palmitate, by the formula:

$$\text{Peak area} \times \frac{M_1 - 28}{M_2 - 28} \times \frac{M_2}{M_1},$$

where M_1 is the mol.wt. of the substance being detected and M_2 is the mol.wt. of methyl palmitate. The percentage of any component is then:

$$\frac{\text{Corrected peak area}}{\text{Total corrected area of all peaks}} \times 100.$$

RESULTS

In Fig. 1, curve A, is shown the result obtained from the gas–liquid chromatograph with a sample of the mixed esters (4·8 mg.) of fatty acids occurring free in human sebum. A total of nineteen peaks in the range from methyl *n*-decanoate to methyl *n*-octadecanoate can be seen. The main components are methyl *n*-dodecanoate, *n*-tetradecanoate and *n*-hexadecanoate and a substance moving in front of methyl *n*-octadecanoate. The saturated acids present in the mixture, determined after conversion of the unsaturated acids into the virtually non-volatile bromo acids by treatment of the mixture with bromine in ether, are shown in Fig. 1, curve *B*. Certain peaks have disappeared and others show a relative decrease, indicating an overlap of peaks due to saturated and unsaturated acids in curve *A*. Correlation of the two results suggests that at least twenty-nine components are present.

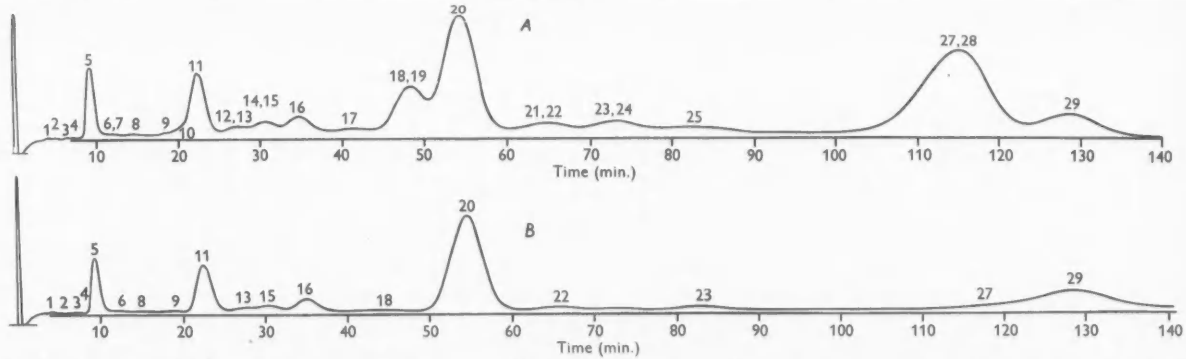

Fig. 1. Fatty acids of human sebum. Curve *A*: separation of 4·8 mg. of methyl esters of the fatty acids occurring free in human sebum. Column length, 4 ft.; stationary phase, high-boiling lubricating oil; temp., 197°; nitrogen pressure, 75·1 cm. Hg; flow rate, 100 ml. of N_2/min. Curve *B*: separation of 4·8 mg. of the same esters after bromination to remove unsaturated acids. Conditions as for *A*. Peaks in order of appearance (as methyl esters): (1) *n*-decanoic; (2) branched-chain saturated C_{11} acid; (3) *n*-undecanoic; (4) branched-chain saturated C_{12} acid; (5) *n*-dodecanoic; (6) highly branched saturated C_{13} acid; (7) mono-unsaturated C_{13} acid; (8) *n*-tridecanoic; (9) simple branched-chain saturated C_{14} acid; (10) mono-unsaturated C_{14} acid; (11) *n*-tetradecanoic; (12) diunsaturated C_{15} acid overlapping (13) highly branched saturated C_{15} acid; (14) mono-unsaturated C_{15} acid overlapping (15) simple branched-chain saturated C_{15} acid; (16) *n*-pentadecanoic; (17) diunsaturated C_{16} acid; (18) simple branched-chain saturated C_{16} acid overlapping (19) mono-unsaturated C_{16} acid (not palmitoleic); (20) *n*-hexadecanoic; (21) diunsaturated C_{17} acid overlapping (22) highly branched saturated C_{17} acid; (23) simple branched-chain saturated C_{17} acid overlapping (24) mono-unsaturated C_{17} acid; (25) *n*-heptadecanoic; (26) poly-unsaturated C_{18} acid; (27) simple branched-chain saturated C_{18} acid overlapping (28) mono-unsaturated C_{18} acid (oleic acid), and (29) *n*-octadecanoic. Ordinates represent gas densities throughout.

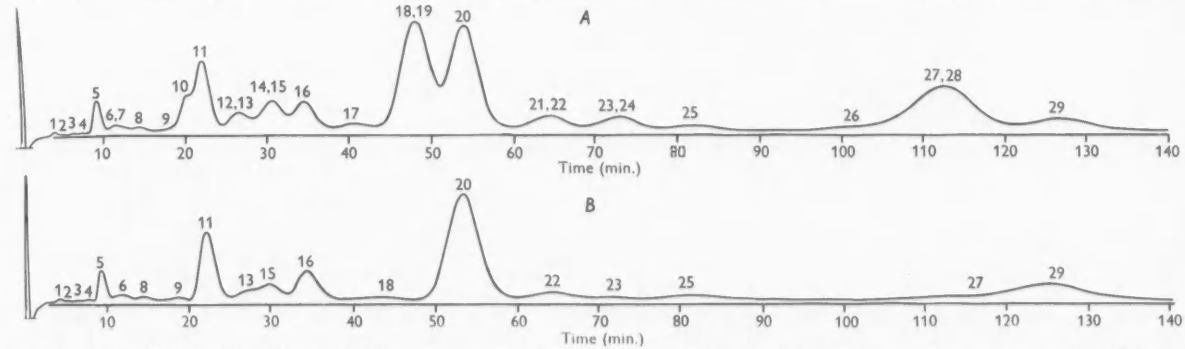

Fig. 2. Fatty acids of human sebum. Curve *A*: separation of 4·8 mg. of the methyl esters of fatty acids existing combined in human sebum. Conditions as for Fig. 1. Curve *B*: separation of 4·8 mg. of the same methyl esters after removal of unsaturated acids by bromination. Peak identification as for Fig. 1.

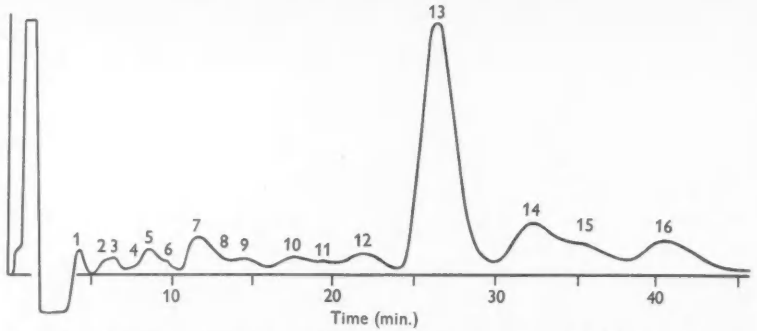

Fig. 3. Separation of methyl esters of free short-chain acids in human sebum. Total load 12 mg.; column conditions as Fig. 1, except that nitrogen pressure is 13 cm. Hg and nitrogen flow rate 14·5 ml./min. Peak identification: (1) branched-chain C_7 acid; (2) *n*-octanoic; (3) highly branched C_9 acid; (4) *n*-nonanoic; (5) highly branched C_{10} acid; (6) simple branched-chain C_{10} acid; (7) *n*-decanoic; (8) highly branched C_{11} acid; (9) simple branched C_{11} acid; (10) *n*-undecanoic; (11) highly branched C_{12} acid; (12) simple branched C_{12} acid; (13) *n*-dodecanoic; (14) highly branched C_{13} acid; (15) simple branched C_{13} acid; (16) *n*-tridecanoic. Peak 13 corresponds with peak 5 in Fig. 1.

Table 1. *Approximate analysis of the acids present in the free state in human sebum in the range* C_7–C_{18}

Acid	Percentage
Saturated *n*-C_7	Trace (i.e. <0·05%)
Branched saturated C_8	Trace
Saturated *n*-C_8	Trace
Branched saturated C_9	Trace
Saturated *n*-C_9	Trace
Branched saturated C_{10}	Trace
Saturated *n*-C_{10}	0·08
Branched saturated C_{11}	Trace
Saturated *n*-C_{11}	Trace
Branched saturated C_{12}	Trace
Saturated *n*-C_{12}	3·64
Branched saturated C_{13}	0·25
Saturated *n*-C_{13}	0·25
Mono-unsaturated C_{14}	0·76
Branched saturated C_{14}	0·32
Saturated *n*-C_{14}	6·35
Diunsaturated C_{15}	0·28
Highly branched saturated C_{15}	0·54
Mono-unsaturated C_{15}	0·64
Branched saturated C_{15}	0·67
Saturated *n*-C_{15}	2·27
Mono-unsaturated C_{16}	9·05
Branched saturated C_{16}	0·29
Saturated *n*-C_{16}	24·2
Diunsaturated C_{17}	0·69
Highly branched saturated C_{17}	1·31
Mono-unsaturated C_{17}	1·62
Branched saturated C_{17}	0·73
Saturated *n*-C_{17}	1·07
Mono-unsaturated C_{18}	35·6
Branched saturated C_{18}	1·8
Saturated *n*-C_{18}	8·0

It was pointed out in an earlier publication (James & Martin, 1956*a*) describing the application of the gas–liquid chromatogram to the long-chain fatty acids that the time of emergence of a peak can be used in structure identification. On this basis it is possible to give tentative identifications of the acids present in this natural mixture. These are as follows: peaks 1, 3, 5, 8, 11, 16, 20, 25 and 29: the homologous series of saturated straight-chain acids of chain lengths from C_{10} to C_{18}; peaks 2, 6, 13 and 22: a series of highly branched odd-numbered saturated acids of chain lengths C_{11}, C_{13}, C_{15} and C_{17}; peaks 7, 12, 17, 21 and 26: unsaturated acids containing probably two double bonds and assignable to straight-chain acids of lengths C_{13}, C_{15}, C_{16}, C_{17} and C_{18}; peaks 9, 15, 18, 23 and 27: branched-chain saturated acids (the branch being probably a single methyl group) of lengths C_{14}, C_{15}, C_{16}, C_{17} and C_{18}; peaks 10, 14, 19, 24 and 28: mono-unsaturated acids, probably straight-chain, of lengths C_{14}, C_{15}, C_{16}, C_{17} and C_{18}.

The existence of these series is shown more clearly in Fig. 2, curves *A* and *B*, which represent the result obtained from samples of the methyl esters of the combined fatty acids from human sebum. Although the relative proportions of the acids are different the general picture is the same as that obtained with the free fatty acids. Conclusive identification of the minor components must await their isolation and degradation. There is, however, no doubt that the C_{16} unsaturated acid is not palmitoleic acid (hexadec-9-enoic acid), since its peak position is different from that due to palmitoleic acid. The C_{18} unsaturated acid lies in the position expected for methyl oleate. The existence of very small amounts of shorter-chain acids in sebum was demonstrated by using a higher load and running the chromatogram more slowly. Fig. 3 gives the result obtained under these conditions; at least sixteen components are present ranging in chain length from C_7 to C_{13}. The general pattern is

very similar to that given by the higher acids. The closeness of the peaks renders identification by position more difficult, but it can be seen that in the range C_7–C_{13} a number of simply branched and more highly branched acids exist, similar to those in the range C_{12}–C_{18}.

The straight-chain saturated acids present can be assigned to the following peaks: (4) *n*-nonanoic; (7) *n*-decanoic; (10) *n*-undecanoic; (13) *n*-dodecanoic; (16) *n*-tridecanoic.

The relative amounts of fatty acids obtained by measurement of peak areas are given in Table 1 for the acids occurring free in human sebum.

DISCUSSION

The results presented here extend and corroborate those of other workers. The higher resolution of the technique has shown not only the series of acids ranging from *n*-heptanoic to *n*-octadecanoic acid demonstrated by Weitkamp *et al.*, but also two series of branched-chain acids. The first of these is assumed from its position on the chromatogram to consist of highly branched acids (though the possibility of cyclic structures should not be overlooked) and is restricted to the odd-number acids. The second series is considered to possess only a simple methyl branch and occurs with both odd and even numbers of carbon atoms. Both mono- and di-unsaturated acids of a variety of chain lengths are present.

Chemical degradation of the C_{16} mono-unsaturated acid by Weitkamp *et al.* showed it to be anomalous in that the double bond was located in the 6:7-position and not in the 9:10-position of the normally encountered palmitoleic acid. The chromatographic position of the C_{16} acid found here is not inconsistent with such an unsaturated acid. The presence of an unsaturated C_{18} acid in the position expected for methyl oleate agrees with the results of Weitkamp *et al.* (1947), Engman & Kooyman (1934) and Ricketts *et al.* (1951), all of whom identified oleic acid in human sebum.

The relative amounts of acids present as estimated by measurement of peak areas (Table 1) vary only slightly from those reported by Weitkamp *et al.* (1947). In particular, only approximately 25% of the free C_{16} acids are unsaturated, as against the 50% previously reported; such a relationship exists only in the combined fatty acid fraction (Fig. 2, curve *A*). Smaller amounts of the short-chain acids than the figures given by Weitkamp may be accounted for by losses due to solvent evaporation in the working up of the sebum samples.

No attempt has been made to extend the analysis to acids of chain length greater than C_{18}, as such acids move very slowly under the conditions described and emerge at low concentration in broad bands. However, clear indications were found of the existence of branched-chain C_{19} acids, but no conclusions were drawn as to their structure. Extension of this analytical technique to the C_{19}, C_{20} and C_{22} acids will necessitate higher column temperatures.

SUMMARY

1. The fatty acids from human forearm sebum have been analysed by gas–liquid chromatography of their methyl esters.

2. Both odd- and even-numbered straight-chain fatty acids have been shown to be present as well as two types of branched-chain odd-numbered saturated fatty acids.

3. The principal unsaturated fatty acids contain 14, 16 and 18 carbon atoms.

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The Sulphur Requirements of the Erythrocytic Form of *Plasmodium knowlesi*

By J. D. FULTON and P. T. GRANT
The National Institute for Medical Research, Mill Hill, London, N.W. 7

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Until the discovery by Knowles & Das Gupta (1932) of a malaria parasite virulent for rhesus monkeys, the study of mammalian malaria in the laboratory was seriously hampered. This parasite, which is mildly infective to man, was named *Plasmodium knowlesi* by Sinton & Mulligan (1932).

Although extensive studies have been made on the culture and metabolism of the erythrocytic form of *P. knowlesi* (for reviews see McKee, 1951 and Fulton, 1951), little is known about the extent to which the parasite can satisfy its nutritional requirements from the cytoplasmic content of its host cell, the erythrocyte. The growth of the parasite in the erythrocyte is associated with a reduction in the haemoglobin content and the formation of parasite pigment. This pigment has been identified as haematin (Morrison & Anderson, 1942; Rimington & Fulton, 1947) and is derived from the haem moiety of globin, the total pigment (haem plus haematin) content of the parasitized cell remaining constant (Ball, McKee, Anfinsen, Cruz & Geiman, 1948). In contrast, the total nitrogen of the infected erythrocyte decreases, and this loss has been attributed to the hydrolysis of globin by the parasite and the utilization of about half the products of hydrolysis for the synthesis of its own protein (Morrison & Jesky, 1948; see also McKee, 1951). In spite of this apparent excess of available amino acids, an additional source of methionine appears to be essential for the growth of *P. knowlesi* both *in vitro* and *in vivo* (McKee, Geiman & Cobbey, 1947; Geiman & McKee, 1948). The reasons for this specific requirement are not known, but it has been suggested that the low methionine content of haemoglobin may make it necessary for the parasite to draw on the plasma for additional supplies of this amino acid (McKee, 1951).

The present paper is concerned with the sulphur-containing compounds within the monkey erythrocyte, and their utilization by the parasite for the synthesis of its own protein.

EXPERIMENTAL

Source of plasmodia. The strain of *P. knowlesi* used was obtained from Dr D. G. Davey of I.C.I. Pharmaceuticals Ltd., Wilmslow, Cheshire. It had recently been isolated in Malaya and proved very virulent for *Macacus rhesus* hosts in which it was passaged. A chronic infection in this host was maintained by the method of Edeson & Davey (1953), with atebrin. The development cycle is of 24 hr. duration and most of the parasites are at the same stage of growth at any one time (synchronous infection).

Cell counts. Total red-cell counts were made in a Thoma–Zeiss haemocytometer. The percentage of parasitized cells was determined in preparations stained by the Leishman method. Parasites freed from host cell by treatment with saponin were also counted in the haemocytometer. The percentage of reticulocytes was determined in a fresh red-cell suspension stained supravitally with 1% (w/v) Brilliant Cresyl Blue in 0·6% (w/v) saline.

Labelled compounds. DL-[^{35}S]Methionine was obtained from the Radiochemical Centre, Amersham. DL-[^{35}S]-Cystine was synthesized as described by Arnstein & Grant (1954). DL-[^{35}S]Cysteine hydrochloride was prepared from DL-[^{35}S]cystine as described by Lucas & Beveridge (1940). Before use, the hydrochloride was converted into the free amino acid by neutralizing an ethanolic solution with pyridine. The precipitate of DL-[^{35}S]cysteine was filtered off and well washed with ether. On titration with 0·01 N iodine, this sample required 97% of the theoretical amount calculated for the free thiol compound.

In vitro *incubation.* (*A*) Monkey blood (100 ml.) containing 18% infected cells (all half-grown parasites) was obtained by cardiac puncture, with heparin added as anticoagulant. This volume of blood was mixed with 20 ml. of a synthetic nutrient medium so that the final concentration of the components (salts, sugars, amino acids, vitamins and nucleic acid bases) in the suspension was that given by Geiman (1949). The blood suspension was dispensed in 10 ml. amounts into 50 ml. conical flasks and rocked (12 rev./min., rocking arc 30°) for 4 hr. at 37°. During this time a slow stream of a water-equilibrated mixture of CO_2 in air (5:95, v/v) was passed over each flask. At the end of the incubation period, the parasite material and haemoglobin were isolated as described below.

(*B*) From monkey blood (20% of erythrocytes infected with half-grown parasites), a concentrated suspension of infected erythrocytes (73% parasitized) was obtained by the procedure described by Geiman, Anfinsen, McKee, Ormsbee & Ball (1946). This suspension was centrifuged at 2000 rev./min., (30 cm. head diameter) for 10 min., the plasma removed and 5 ml. of the sedimented cells resuspended in 40 ml. of a serum-containing nutrient medium (Geiman, 1949), in which the amino acid mixture had been replaced by DL-[^{35}S]cysteine (0·033 m-mole) and non-isotopic DL-methionine (0·033 m-mole). Another 5 ml. of the sedimented cells was resuspended in a similar medium in which DL-[^{35}S]methionine (0·033 m-mole) and non-isotopic DL-cysteine (0·033 m-mole) were present. Each of

these suspensions was incubated at 37° in five flasks containing 8 ml. amounts as described above. At intervals, one flask from each group was removed, the cells were isolated by centrifuging and washed three times in isotonic saline containing 0·2% (w/v) glucose, and non-isotopic cysteine and methionine in the concentrations given above. The washed sedimented cells were then freeze-dried. Crude protein was isolated and oxidized with performic acid as described below.

Production of ^{35}S*-labelled erythrocytes and their transfusion into a monkey parasitized with* Plasmodium knowlesi. The erythrocytes of a small monkey (2¼ kg.) were checked for compatibility with the plasma of a larger one (3¼ kg.). Reticulocytosis was induced in the smaller animal by daily bleeding, a total of 50 ml. being removed in a period of 4 days. At 41 hr. after the final bleeding, when approximately 16% of the total red cells were reticulocytes, a single dose of DL-[^{35}S]methionine (5 mg., 250 μc) was injected intraperitoneally. The incorporation of radioactivity into the red cells was followed and was found to reach a maximum 8 days after the injection of the labelled material.

During this time, the larger monkey was infected with *P. knowlesi*. When approx. 10% of the erythrocytes contained nearly full-grown parasites the smaller monkey was anaesthetized (12 days after injection of the labelled material) and bled by cardiac puncture into a mixture of 2% (w/v) sodium citrate in 1% (w/v) NaCl. The erythrocytes were centrifuged at 2000 rev./min. (30 cm. head diameter) for 10 min. and washed 4 times with 0·9% (w/v) NaCl containing 0·2% (w/v) glucose. The final suspension was centrifuged, the supernatant removed and 25 ml. of the packed erythrocytes were resuspended in an equal volume of the glucose–saline solution. This suspension was slowly and continuously injected over a 30 min. period into a leg vein of the parasitized monkey. During the whole of this period the animal was lightly anaesthetized with ether.

Isolation of malaria parasites and parasite protein. Usually when about 40% of the red cells were infected, the parasitized monkey was anaesthetized with ether and 1 ml. of a 1% (w/v) heparin solution injected intravenously. The animal was bled by cardiac puncture into citrated saline containing 0·2% (w/v) glucose. The vascular system was then perfused with saline by means of a cannula in the aorta and the perfusing liquid collected by a cannula in the inferior vena cava.

The total blood was centrifuged at 2000 rev./min. for 10 min., the diluted plasma was retained and the blood cells were resuspended in an equal volume of fresh citrated saline. This suspension was slowly drawn through columns of dry Solka-Floc (Johnsen, Jörgensen and Wettre, London), 2 g./75 ml. of suspension, with a negative pressure in the receiving vessel. Such a procedure removes practically all leucocytes from the blood.

The parasites were then isolated by lysis of the erythrocytes with saponin as described by Christophers & Fulton (1939). After washing the parasites three times in saline, samples were taken for microscopic examination and the remainder was freeze-dried.

In early experiments, pigment was removed from the parasite by extraction with ethanolic KOH (see Deane, 1945), but subsequently cleaner preparations were obtained by extraction with HCl–acetone mixtures. For example, 200 mg. of parasite material, with a few small glass beads to aid breaking up the organism, was shaken for 30 min. on a Microid shaker (Griffin and Tatlock, London) with three successive amounts (10 ml.) of HCl–acetone (1 ml. of 2N-HCl in 100 ml. of acetone). The residue was extracted with two successive amounts (10 ml.) of 5% (w/v) trichloroacetic acid at 90° for 10 min. Finally the pale buff-coloured solid was washed with ethanol and ether, and dried. Yield, 124 mg.

Isolation of globin and total intracellular protein from lysed erythrocytes. After saponin lysis of the erythrocytes and removal of the malarial parasites, the intracellular proteins of the erythrocytes were obtained from the supernatant in the following way: red-cell membranes were agglutinated by passing CO_2 into the solution for 60 min. (Parpart, 1942) and sedimented by centrifuging at 10 000 rev./min. in an angle centrifuge (about 9000 *g*) for 20 min. The clear red solution which remained was dialysed against two successive amounts (100 vol.) of physiological saline at 0° for 24 hr. For isolation of total protein from the residue in the sac a portion was treated at 90° with trichloroacetic acid for 10 min. at a final concentration of 10% (w/v). The precipitate was centrifuged and re-extracted with 10% (w/v) trichloroacetic acid, washed with ethanol and ether, and dried. Globin was isolated from another portion by the acid–acetone technique of Anson & Mirsky (1929–30).

Purification of isotopic protein samples. Before hydrolysis or direct radioassay of the protein samples, it was necessary to eliminate the possibility of adsorption or attachment by disulphide linkage of free sulphydryl compounds to the protein. For this purpose, each sample (150 mg.) was oxidized in performic acid (10 ml. of 98%, w/v, formic acid and 2 ml. of 100 vol. H_2O_2) and protein in the clear solution was reprecipitated with trichloroacetic acid (see Peterson & Greenberg, 1952). In this procedure, cystine is oxidized to cysteic acid and methionine to its sulphone.

Dilution of labelled protein and the isolation of cystine and methionine. From an infected monkey usually only 100–400 mg. of isotopic parasite protein was obtained. Before hydrolysis, such samples were accurately diluted by weight with non-isotopic protein isolated in an identical manner from mature parasites.

The protein samples (usually about 0·8–1·0 g.) were hydrolysed with 6N-HCl in sealed tubes at 105° for 18 hr. Hydrochloric acid was removed by repeated evaporation *in vacuo* and the residue kept overnight *in vacuo* over solid KOH. The residual solid was taken up in 5 ml. of water and the filtered solution was then applied to a column of Zeo-Karb 225 (Permutit Co., London) in the hydrogen form (750 g./g. of original protein hydrolysed). The column was developed with 1·5N-HCl, the effluent being collected in 7 ml. fractions, and the amino acids were detected by paper chromatography with phenol–NH_3 soln. as developing solvent. Cysteic acid is the first amino acid to be eluted (Arnstein & Grant, 1954), methionine sulphone being eluted after glutamic acid.

The fractions containing cysteic acid were pooled and evaporated to dryness *in vacuo*, those containing methionine sulphone being similarly treated. Each residue was redissolved in distilled water (2 ml.) and inorganic sulphate precipitated by the addition of a solution of 0·22M benzidine dihydrochloride (1 ml.) followed by anhydrous acetone (2 ml.). After 4 hr. at 4°, the small precipitate of benzidine sulphate was filtered off, and the filtrate evaporated to

dryness. Cysteic acid and methionine sulphone were converted into inorganic sulphate by digestion with the reagent described by Pirie (1932). Because of the recorded difficulty in oxidizing methionine with this reagent (Callan & Toennies, 1941) all samples of methionine sulphone were digested for 72 hr. The inorganic sulphate was precipitated with benzidine by a procedure similar to that described by Simpson & Tarver (1950).

Radioactivity measurements. All measurements were carried out with a helium-filled, bell-shaped end-window Geiger–Müller counter, using 'infinite thickness' samples, the substances being mounted on 1 sq.cm. polythene disks (Popják, 1950). All counts were compared with those of a [^{14}C]Perspex reference standard obtained from the Radiochemical Centre, Amersham, and were corrected for decay by direct comparison with a subsidiary ^{35}S standard sample. The standard error of all determinations was $\pm 5\%$. For assay of ^{35}S as benzidine sulphate, the substance was mounted by filtration on filter paper (Whatman no. 50) on 1 sq.cm. stainless-steel perforated disks. The amount of benzidine sulphate present on a disk was determined by titration at 100° against 0·01 N-NaOH with phenol red as an internal indicator. The amount usually present was 4–10 mg., and all radioactivities were corrected to 'infinite thickness' by means of a correction factor. This factor was obtained from a chart made by plotting the observed radioactivities of a standard benzidine sulphate sample mounted in amounts of 2–50 mg. as described above.

Isolation and determination of glutathione. Erythrocyte glutathione was isolated via the cuprous mercaptide, essentially as described by Dimant, Landsberg & London (1955).

For the estimation of glutathione, about 4 ml. of blood was centrifuged and the white-cell layer removed by pipette. The red cells were resuspended in saline to give the original blood volume, a small sample was taken for cell counts and 1 ml. samples were used to estimate glutathione by the method of Hardin, Valentine, Follette & Lawrence (1954).

Total sulphur. This was determined by the gravimetric wet-combustion method (Carius) as described by Niederl & Niederl (1941).

Determination of cystine and methionine. These amino acids were determined directly in globin by the technique of Evans (1945), in which nitric acid is used as a selective oxidizing reagent. The standard error in this estimation is about $\pm 10\%$.

RESULTS

Preparation of malaria parasites essentially free of the host erythrocyte. After saponin lysis of infected erythrocytes and extensive washing of the parasites, stained preparations (Leishman) appear normal and no erythrocytic membrane is visible (cf. Christophers & Fulton, 1939). Ultraviolet microscopy, however, suggests that some of the parasites may still be attached to the erythrocyte membrane, but this was not studied further since calculation showed that the mass of these membranes relative to that of the parasite was insignificant.

Amino acid composition of the parasite protein. No unusual amino acids were detected when hydrolysates of parasite protein were analysed on columns of Zeo-Karb 225. Cystine and methionine were the only sulphur-containing amino acids found to be present.

Conversion of methionine sulphur into cystine sulphur by Plasmodium knowlesi in vitro. When a suspension of infected erythrocytes was incubated with a chromatographically pure sample of DL-[^{35}S]methionine, this amino acid in the parasite protein became highly radioactive, while the cystine contained significant radioactivity (Table 1).

The cystine of the host-cell globin was slightly radioactive, and this may have served as a source of labelled cystine for the parasite. However, when a non-infected suspension of monkey blood (containing 6% of reticulocytes) was incubated with DL-[^{35}S]methionine in an identical manner to that described in Table 1, globin methionine contained 0·27 μC/m-mole, but no significant radioactivity was found either in globin cystine or in the intracellular free cystine and glutathione. This clearly indicates that there is no detectable conversion of methionine sulphur into cystine sulphur by erythrocytes or reticulocytes under these conditions.

It is therefore concluded that the radioactivity in cystine of the parasite protein could arise only by a conversion of methionine into cystine by the parasite. In this case, the small radioactivity in the globin cystine (Table 1) remains to be explained. In a red-cell population, only reticulocytes are known to incorporate labelled amino acids into haemoglobin (Borsook, Deasy, Haagen-Smit, Keighley & Lowy, 1951), so that the diffusion of [^{35}S]cystine from parasitized cells into reticulocytes may account for this observed incorporation of [^{35}S]-cystine into haemoglobin.

Table 1. *Conversion of methionine sulphur into cystine sulphur by* P. knowlesi in vitro

A suspension of monkey blood and synthetic nutrients was incubated for 4 hr. with DL-[^{35}S]methionine (0·58 mg., 25 μC) as described in the Experimental section. This suspension contained 6% of infected red cells (Leishman stain). 10% of the red cells stained supravitally with Brilliant Cresyl Blue, i.e. parasitized red cells and reticulocytes, so that by difference 4% of the red cells are non-parasitized reticulocytes. At the end of the incubation period, cystine and methionine were isolated from the protein of the parasite and from globin as described in the Experimental section.

Amino acid	Molar radioactivity of amino acid (μC/m-mole) in Parasite protein	Globin
Cystine*	0·15	0·0024
Methionine	6·49	0·049

* Calculated as μC/m-mole of cysteine.

Utilization of ^{35}S*-labelled haemoglobin by* Plasmodium knowlesi. To study the utilization of ^{35}S-labelled haemoglobin by *P. knowlesi*, labelled erythrocytes were prepared and injected into a parasitized monkey infected with large forms of the parasite which had completed about three-quarters of their cycle of development. This allowed time for complete mixing of the labelled cells with unlabelled cells already present in the monkey, before a new invasion of erythrocytes by merozoites from the ruptured mature schizonts took place. The completion of the next cycle of development of the parasites within labelled and unlabelled red cells occurred 29 hr. after the injection of the labelled cells. Re-invasion of fresh red cells again occurred. Near the end of the 24 hr. developmental period of this new generation of parasites, that is after two growth cycles had occurred in the presence of the labelled erythrocytes, the animal was bled out under anaesthesia and the parasites were isolated.

It was first necessary to determine whether the ratio of ^{35}S-labelled red cells to normal red cells remained constant over the experimental period. From Table 2 it can be seen that at first there was an increase in the number of red cells as a result of the injection of labelled cells, and then in the interval between 1·5 and 24 hr. there is an observed decrease of 8·3 %, of which not less than 5·2 % was due to rupture of the red cells by parasites. Similarly, the observed decrease of 12 % in the red-cell count in the period of 24–47 hr. can be largely attributed to destruction of cells by the parasites, in this case not less than 9·2 %. Thus there appears to be some destruction of red cells besides that attributed to the parasite, but in our opinion fluctuations in the parasite population as a result of host defences and inherent difficulties in counting red cells may make such differences of limited significance. Since the specific radioactivities of samples of the haemoglobin measured at 1·5, 24 and 47 hr. were similar, it is evident that there has not been a selective destruction of the labelled red cells. Moreover, these similar radioactivities would indicate that the production of reticulocytes has not markedly affected the proportion of labelled to unlabelled red cells. For these reasons it was concluded that the ratio of ^{35}S-labelled erythrocytes to

Table 2. *Experimental data on a monkey parasitized with* P. knowlesi *after the injection of* ^{35}S*-labelled erythrocytes*

Time after injection of labelled erythrocytes (hr.)	Erythrocytes (millions/mm.^{3})	Parasitized erythrocytes (%)	Stages of parasite growth (% of infected erythrocytes*)				Specific radioactivity of haemoglobin (μc/g.)
			Rings	Tropho-zoites	Early schizonts	Mature schizonts	
0	5·1	7·3	0	1	20	79	—
1·5	7·2	5·2	0	0	5	95	0·018
3	—	5·5	18	0	0	82	—
6	—	8·9	93	2	0	5	0·016
24	6·6	9·2	0	0	11	89	—
29	—	26·7	98	0	0	2	—
47	5·8	23·1	0	0	23	77	0·017

* Percentage of erythrocytes infected with asexual forms of *P. knowlesi*.

Table 3. *Utilization by* P. knowlesi *of erythrocyte protein labelled with* ^{35}S *cystine and methionine*

The course of the infection in Expt. 1, in which two growth cycles of the parasite took place in ^{35}S-labelled erythrocytes, is detailed in Table 2. Other procedures are described in the Experimental section. Expt. 2 was essentially similar except that only one growth cycle of the parasite took place within the experimental period.

Amino acid or peptide	Expt. no.	Molar radioactivity of amino acid or peptide (μc/m-mole) in: Parasite protein (*A*)	Globin (*B*)	Total intracellular protein of the erythrocyte	Erythrocytes	$A/B \times 100$
Cystine*	1	0·042	0·064	0·060	—	65·6
	2	0·013	0·018	—	—	72·2
Methionine	1	0·078	0·10	0·097	—	78·0
	2	0·082	0·15	—	—	54·7
Reduced glutathione	1	—	—	—	0·013	—
	2	—	—	—	0·005	—

* Calculated as μc/m-mole of cysteine.

normal erythrocytes remained essentially constant over the experimental period.

At the end of the experimental period (47 hr.) the protein of the parasite, the haemoglobin and the total intracellular-erythrocytic protein contained significant radioactivity. The molar radioactivities of cystine and methionine isolated from these samples are shown in Table 3. It will be seen from these results that the ratio of the molar radioactivity of methionine in the parasite protein to that in globin is greater in Expt. 1 than that found in Expt. 2. At least a part of this difference may be accounted for by the following consideration. In one growth cycle, each parasite will divide into 4–16 daughter cells, with an average of 10 (Singh & Nair, 1954). Thus the final parasite material isolated would have derived more of its mass from the unlabelled parasite present at the beginning of Expt. 2, in which only one growth cycle took place, than that isolated where two consecutive growth cycles occurred.

It will be noted in Expt. 2 that the ratio of the molar radioactivity of cystine in the parasite protein to that of globin cystine is greater than that for methionine, whereas in Expt. 1 the opposite is true. The *in vitro* conversion of methionine sulphur into cystine sulphur has already been indicated, so that a similar conversion *in vivo* may account for the difference in these two experiments since the radioactivity of globin methionine was some ten times greater than that of cystine in Expt. 2, but less than twice that in Expt. 1.

Determination of sulphur compounds contained in the parasite and in an equal number of normal erythrocytes. In view of the important part played by haemoglobin in the nutrition of the parasite, it is of some consequence to determine whether the host cell contains sufficient potentially available sulphur compounds to supply the entire requirements of a parasite. For this purpose the sulphur content and sulphur-containing compounds have been estimated in a known number of mature parasites (Table 4) and the same number of normal erythrocytes (Table 5).

Table 4. *Mass and sulphur content of a known number of parasites*

Expt. 1. A monkey (2¼ kg.) with 6% of its erythrocytes parasitized was injected intraperitoneally with an aqueous solution of DL-[^{35}S]cysteine (35 mg., 20·2 μc) and the injection was repeated 24 hr. later. At 19·5 hr. after the last injection, when approximately 47% of the erythrocytes were infected with mature forms of the parasite, the animal was bled out and the parasites were isolated as described in the Experimental section. The washed, free, parasites were suspended in isotonic saline and the volume was made up to 50 ml. 48 ml. of this suspension was centrifuged at 4000 rev./min. for 10 min. in an angle centrifuge, the supernatant was removed and the residue freeze-dried. The remainder of the suspension was used to make stained smears and to determine the number of parasites/ml. Expt. 2 was a preparation of parasites from a monkey which had received no isotope.

Material	Expt. no.	Sulphur content (%)	Radioactivity (μc/mg. of sulphur)	Values for 10^{11} parasites: Mass (g.)	Values for 10^{11} parasites: Total sulphur (mg.)
Whole dried parasite material	1	0·54	—	1·01	5·45
	2	0·49	—	1·27	6·22
Parasite protein	1	0·90	0·0014	0·42	3·78
	2	0·85	—	0·54	4·59
Amino acids of the parasite protein: Cystine	1	—	0·0022	—	—
Amino acids of the parasite protein: Methionine	1	—	Nil	—	—

Table 5. *Sulphur content and sulphur-containing amino acids in the globin of monkey erythrocytes*

Monkey blood (about 25 ml.) was freed from leucocytes by centrifuging, the erythrocytes were washed 3 times in isotonic saline and the final suspension was made up to 25 ml. The number of erythrocytes/ml. was determined and the red cells in a known volume were lysed. The lysate was freed from red-cell membranes and globin isolated from the residual solution by the procedures given in the Experimental section. The protein was oxidized with performic acid, cystine and methionine being estimated in this sample by the differential-oxidation technique of Evans, 1945.

Material	Sulphur (%)	Amino acid (g./100 g. of protein)	Values per 10^{11} erythrocytes for: Total amino acid (mg.)	Total mass (g.)	Total sulphur (mg.)
Globin	0·62	—	—	2·87	17·79
Globin, oxidized with performic acid	0·59	—	—	2·70	15·93
Cystine	—	0·8*	21·5	—	5·7
Methionine	—	1·4*	38·0	—	8·2

* Mean value of three estimations.

From the results of Expt. 1 in Table 4, it is possible to calculate the cystine and methionine content of the parasite protein. This method of calculation is valid for this particular experiment since it has been shown that cystine and methionine are the only sulphur-containing compounds in the protein and that cystine sulphur is not converted into methionine sulphur when DL-[^{35}S]cysteine is administered to an infected monkey. The specific radioactivity of the parasite protein was 0·0014 μc/mg. of sulphur, which was entirely due to its cystine content. Since the specific activity of cystine isolated from this protein was 0·0022 μc/mg. of sulphur, then 1 mg. of protein sulphur must contain 0·64 mg. of cystine sulphur of this radioactivity, and, by difference, 0·36 mg. of non-isotopic methionine sulphur. From the sulphur content of the protein (0·9 %) it may be calculated that 100 mg. of parasite protein contains 2·18 mg. of cystine and 1·67 mg. of methionine.

Of the total sulphur content of the parasite, some 70 % appears to be associated with the sulphur-containing amino acids of the protein (Table 4). The non-protein residue (substances soluble in hot 5 % (w/v) trichloroacetic acid) contained inorganic sulphate, but attempts to identify organic sulphur compounds by paper chromatography were unsuccessful.

The total amount of intracellular protein contained in a known number of normal erythrocytes was determined in various ways. Thus, protein precipitation from a membrane-free lysate of red cells by 10 % (w/v) trichloroacetic acid, by heat coagulation at pH 4·5, or by an acetone precipitation, all gave similar results with a mean mass of 3·1 g. of protein/10^{11} cells. A slightly lower value (2·87 g.) was obtained for total intracellular globin by the relatively more specific method of Anson & Mirsky (1929–30). The amounts of cystine and methionine in globin oxidized by performic acid (Table 5) are similar to those obtained for globins of other mammalian species (Beach, Bernstein, Hummel, Williams & Macy, 1939). However, it is to be noted that the values given in Table 5 account for only about 85 % of the total sulphur in the protein. Indeed, by the method used, only some 83–89 % of the total sulphur could be similarly accounted for in various samples of haemoglobin and globin, a fact which may be associated with the difficulty involved in the complete oxidation of methionine (Callan & Toennies, 1941). The results shown in Table 6, in which values for recognized sulphur components of the parasite and the erythrocyte are compared, indicate that there is a large excess of cystine, methionine and glutathione in the red cell which is potentially available to the parasite.

A comparison of the uptake of ^{35}S-labelled cysteine and methionine into parasitized erythrocytes in vitro. Since the parasite *in vivo* is capable of utilizing free cysteine of the plasma (Table 4), a comparison was made of the uptake of ^{35}S-labelled cysteine and methionine from the medium by parasitized cells *in vitro* (Fig. 1). The parasite protein contains approximately twice as much cysteine as methionine (Table 6), so that the low incorporation of cysteine relative to that of methionine was not to be expected if both amino acids were freely permeable to the erythrocyte. One possible explanation is that the concentration of cysteine decreases over the experimental period due to its oxidation to cystine. The latter compound might not be permeable to the red cell or readily utilized by the parasite. However, the amount of free thiol compound remaining in the medium after 3 hr. incubation, as judged by the method of Hardin *et al.* (1954), was 73 % of that found in the beginning of the experiment. In the absence of added cysteine, infected erythrocytes produce relatively little free thiol compounds in the medium after 3 hr. incubation.

Glutathione content of parasitized erythrocytes. In five samples of monkey blood it has been estimated that there is a mean value of 9·2 mg. of reduced glutathione in 10^{11} monkey erythrocytes (cf. Table 6), a value which is in reasonable agreement with that obtained for human erythrocytes by Hardin *et al.* (1954). Estimations were then made of the glutathione content of erythrocytes parasitized with *P. knowlesi*. For example, during the development *in vivo* of young ring forms to the mature schizont stage the glutathione content of

Table 6. *A comparison of the sulphur content of equal numbers of mature forms of* P. knowlesi *and normal monkey erythrocytes*

For details of the results in this table, see Tables 4 and 5. The determination of glutathionine is described in the Experimental section.

	Sulphur content (mg.) of 10^{11} cells			
		In protein		
	Total	Cystine	Methionine	In glutathione
P. knowlesi free from host erythrocyte	3·78	2·43	1·36	—
Uninfected erythrocytes	15·93	5·7	8·2	1·01
Erythrocytes/parasites	4·21	2·3	6·0	—

Fig. 1. A comparison of the uptake of ^{35}S-labelled cysteine and methionine by parasitized erythrocytes *in vitro*. Identical suspensions of erythrocytes (73% infected with *P. knowlesi*) were incubated for various times with equimolar amounts of either DL-[^{35}S]cysteine (0·22 μc/m-mole) or DL-[^{35}S]methionine (0·72 μc/m-mole) as described in the Experimental section. The observed radioactivities (counts/min. above background for 'infinitely thick' samples of protein, from parasitized erythrocytes, of 1 sq.cm. area) were made comparable by multiplying those of the cysteine experiment by a correction factor. This was obtained by dividing the molar radioactivity of the added methionine by that of the cysteine. □, Methionine experiment; ○, cysteine experiment.

the red cells (14% infected with *P. knowlesi*) decreased from 8·60 to 7·60 mg. of glutathione per 10^{11} erythrocytes, a fall of approximately 11·6%. In a heavier infection (22% of red cells parasitized) the content of the erythrocytes decreased from 8·90 to 7·17 mg. of glutathione/10^{11} cells, a fall of 19·4%.

DISCUSSION

Although free cystine and methionine in the medium or plasma can be used by erythrocytic forms of *P. knowlesi* (Tables 1 and 4, Fig. 1), it has also been established that the parasite utilizes sulphur-containing compounds of the red cell for the major part of its requirements for these amino acids. Thus, after the development of *P. knowlesi* through two growth cycles in ^{35}S-labelled erythrocytes *in vivo*, the cystine and methionine of the parasite protein contained 65·6 and 78·0% respectively of the molar radioactivities of these amino acids found in the globin moiety of haemoglobin (Expt. 1, Table 3). It appears, therefore, that the parasite can hydrolyse globin and utilize the degradation products for the synthesis of its own protein. This would seem likely since it has been shown that extracts of the malaria parasite of the chicken, *Plasmodium gallinaceum*, can hydrolyse haemoglobin slowly and denatured globin more rapidly (Moulder & Evans, 1946), although an attempt to demonstrate a similar proteolytic activity in extracts of *P. knowlesi* was unsuccessful (Ball *et al.* 1948).

Labelled methionine for the synthesis of parasite protein in the experiment mentioned above could only be derived from the protein of the erythrocyte, and so nearly 80% of the parasite's requirements for the amino acid is derived from this source, the remainder presumably being obtained from non-isotopic free amino acid in the plasma. This conclusion is in agreement with the observation that the growth of *P. knowlesi* within the erythrocyte *in vitro* is dependent on a supply of free methionine in the medium (McKee *et al.* 1947). Since globin in an erythrocyte contains 6·0 times as much methionine as the protein of a mature parasite (Table 6), the requirement for an extra-erythrocytic supply of this amino acid indicates that most of the globin methionine must be unavailable to the parasite. This may be occasioned by the rate of globin hydrolysis being insufficient to meet the needs of the growing parasite.

The extent to which cystine in the parasite protein is derived from any one source is, however, difficult to determine. In the *in vivo* experiment, cystine of widely differing radioactivities (Table 3) may be obtained by the parasite from protein or glutathione of the erythrocyte and by a conversion of methionine sulphur into cystine sulphur. In addition, non-isotopic cystine may be available to the parasite from the free amino acid in the plasma. For these reasons, the radioactivity observed in the cystine of the parasite protein in these experiments shows only the net result, so that the extent to which each source contributes to the cystine requirement of the parasite must be determined by other means.

Cystine of the parasite protein contains 65·6% of the molar radioactivity of globin cystine (Expt. 1, Table 3), so that it is reasonable to assume that globin can serve as a source of this amino acid for *P. knowlesi*. For the reasons given above the radioactivity measurements do not, however, give any idea of the importance of this source to the parasite, but an approximation can be made in the following manner. From the results in Table 6 it may be calculated that, for each molecule of haemoglobin hydrolysed, there will be available to the parasite methionine and cystine sulphur in the ratio 1:0·70. It is assumed that the rate of release of methionine from the hydrolysis of haemoglobin is only sufficient to provide 80% of the parasite's requirements for this amino acid, and that all methionine and cystine liberated is available and is utilized by the parasite. Thus, the ratio of methionine to

cystine sulphur released from the globin would be in the ratio $80:(80\times0{\cdot}70)=80:56{\cdot}0$, whereas the ratio required by the parasite for the synthesis of its own protein (see Table 6) would be

$$100:(100\times1{\cdot}79)=100:179.$$

On this basis 56·0/179 (= 31·3 %) of the cystine in the parasite protein could be derived from globin cystine.

The percentage decrease in the amount of reduced glutathione found in a suspension of erythrocytes is correlated with the percentage of erythrocytes infected with mature forms of *P. knowlesi* (see Results section). It is therefore possible that most of the glutathione present in an infected erythrocyte has been utilized during the growth of the parasite within the host cell. The amount of cystine sulphur contained in the glutathione of an erythrocyte has been shown to be about half that found in the protein of a mature parasite (Table 6), so that some 50 % of the cystine requirement of the parasite could be derived from this source.

Of the amino acids, only methionine is required to be present in a serum-containing medium for the growth *in vitro* of *P. knowlesi* (McKee *et al.* 1947), and this would imply that essentially all the parasite's requirements for the other amino acids are obtained from the intracellular contents of the erythrocyte. In the present experiments, [^{35}S]-cysteine injected into an infected monkey was found to be incorporated into the protein of the parasite (Table 4), and for this reason the uptake of ^{35}S-labelled cysteine and methionine into parasitized erythrocytes was compared *in vitro* (Fig. 1). However, there was a surprisingly low uptake of cysteine compared with that of methionine. This fact, together with the estimate made *in vivo* (Table 3) that only about 20 % of methionine in the parasite protein is obtained from free amino acid in the plasma, would indicate that free cysteine in the plasma or medium is a minor source of cystine to the parasite. It may be that the small utilization of plasma cysteine by the parasite (Fig. 1) is occasioned by a restricted penetration of this amino acid into the host erythrocyte. Indeed, there is some evidence (Johnson & Bergeim, 1951) which suggests that while methionine may be freely permeable to human erythrocytes, cystine and presumably cysteine are not.

P. knowlesi under *in vitro* conditions is capable of converting methionine sulphur into cystine sulphur (Table 1). It seems likely, as was indicated in the Results section (Table 3), that this conversion would take place *in vivo*, although the importance of the reaction to the parasite is not known. However, it has been indicated above that some 50 % of the parasite's cystine may be derived from glutathione of the erythrocyte, 30 % from globin cystine and a negligible amount from free cystine in the plasma, so that by difference some 20 % may be obtained by the conversion by the parasite of methionine into cystine. While such estimates can only be regarded as rough approximations, they are in general agreement with the results in Table 3, where in Expt. 1 the parasite underwent two developmental cycles within erythrocytes which contained ^{35}S-labelled globin and glutathione. While the observed radioactivity of cysteine in the parasite protein at the end of this experiment was 0·042 μc/m-mole, it may be calculated that a cysteine sample obtained from the radioactive globin cystine, glutathione and methionine of the parasite in the amounts estimated above would have a radioactivity of 0·041 μc/m-mole. Not quite such good agreement is found between the observed radioactivity (0·013 μc/m-mole) and that calculated (0·024 μc/m-mole) for cysteine in the parasite protein in Expt. 2, Table 3. In this instance, however, only one growth cycle of the parasite took place within ^{35}S-labelled erythrocytes. Thus, the observed radioactivity may be low when compared with that calculated, because the final parasite material would have derived some cystine from that present in the unlabelled parasite at the beginning of the experiment. In one growth cycle, each parasite divides into 4–16 daughter cells (Singh & Nair, 1954), so that the calculated radioactivity (0·024 μc/m-mole) should be reduced to a value 6–25 % lower than that given above (0·018–0·022 μc/m-mole) in order to take this consideration into account. Nevertheless, a difference between the observed and calculated radioactivity of cystine in the protein of the parasite still remains, so that the estimates on which the calculated radioactivity is based can only approximately fit the experimental facts in this particular experiment. The most likely explanation is that the amount of methionine converted into cystine is lower than has been estimated, since cystine available to the parasite from this source contains about 5 times the molar radioactivity of globin cystine and about 16 times that of glutathione (Table 3).

It is concluded that the essential nature of methionine for the growth of *P. knowlesi* is not only occasioned by a requirement for the preformed amino acid, but also as an additional source of cystine which the parasite cannot apparently obtain in sufficient amount from either bound cystine in the erythrocyte or the free amino acid in the plasma.

SUMMARY

1. During the growth of the malaria parasite *Plasmodium knowlesi* in ^{35}S-labelled erythrocytes *in vivo*, it has been shown that about 80 % of the

methionine in its protein is derived from that in the red-cell globin.

2. The conversion by *P. knowlesi* of [^{35}S]-methionine into [^{35}S]cystine has been shown to occur *in vitro*, and this provides a source of cystine sulphur for the parasite in addition to that already present in the haemoglobin and glutathione of the erythrocyte. Some estimates of the extent to which these sources contribute to the cystine requirement of the parasite have been made.

3. Under identical *in vitro* conditions it was found that the uptake of DL-[^{35}S]methionine by parasitized erythrocytes was markedly greater than that of DL-[^{35}S]cysteine.

4. The amounts of methionine and cystine present in the haemoglobin of a monkey erythrocyte are respectively 6·0 and 2·4 times that found in the protein of a mature parasite. The implications of this observation to the nutrition of the parasite have been discussed.

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Products of Hydrolysis of Dinitrophenylproline and *N*-Terminal Proline-peptides with Hydrochloric Acid

By F. S. SCANES and B. T. TOZER

Microbiological Research Department, Porton, Wilts

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During the examination of clupeine for the *N*-terminal amino acid residue (Scanes & Tozer, 1956), considerable difficulty was experienced in isolating ether-soluble dinitrophenyl (DNP) amino acid from the hydrochloric acid hydrolysate in the amount expected. A similar result was obtained by the alternative hydrolytic methods of Hanes, Hird & Isherwood (1952). As proline was suspected as an *N*-terminal amino acid, the substitution of 12N for 6N hydrochloric acid, as recommended by Porter & Sanger (1948) for use with DNP-prolyl proteins, was tried, but without success. Porter & Sanger recovered 50% of the DNP-proline added to globin before hydrolysis, but other workers have reported much lower recoveries, namely, 20% from a collagen hydrolysate

(Bowes & Moss, 1953) and 15% from a gelatin hydrolysate (Courts, 1954) after hydrolysis with 10N and 12N acid respectively.

Salmine, which has been shown by various workers (Velick & Udenfriend, 1951; Landmann, Drake & Dillaha, 1953) to contain proline as the *N*-terminal amino acid, was used for reference purposes. In hydrolytic experiments carried out on DNP-salmine, DNP-proline and DNP-proline admixed with gelatin, we were able to recover only 5–10% of DNP-proline, even after as short a time as 4 hr. in 12N hydrochloric acid at 100°. The main products obtained were 2:4-dinitrophenol and two other DNP derivatives which did not correspond with any of the known DNP-amino acids when examined by column and paper chromatography. The small yields of DNP-proline obtained were rather disconcerting, since DNP-proline has been reported by so many workers as a major hydrolytic product, with up to 50% recovery. However, since the present work was completed, Phillips (1955), who identified proline as an *N*-terminal group of salmine by the examination of the spectral-absorption curve, noted the presence of DNP-proline for only up to 2 hr. in 6N acid hydrolysates, and Fraenkel-Conrat, Harris & Levy (1955) have reported the complete destruction of DNP-proline after hydrolysis with 10N hydrochloric acid at 105° for 2 hr., with the formation of dinitrophenol and two yellow artifacts.

Schramm & Braunitzer (1953) have isolated a number of DNP derivatives from hydrolysates of DNP-proline admixed with horse globin and from the DNP derivative of tobacco mosaic virus. These derivatives included 2:4-dinitrophenol as the major component after full hydrolysis, in addition to traces of prolyl peptides and an unknown yellow compound, although after partial hydrolysis for 2 hr. at 105° Schramm & Braunitzer found some DNP-proline in addition to DNP-prolyl peptides.

With the object of investigating the fate of DNP-proline when it is liberated in acid hydrolysates of DNP-proteins, we have carried out experiments on the hydrochloric acid hydrolysis of DNP-DL-proline and have isolated and identified the major hydrolytic products.

The DNP-hydrolytic products were examined by paper chromatography in four systems which were selected so that, by suitable choice of a combination of systems, the individual hydrolytic products of DNP-proline and the potential DNP-amino acids from a DNP-clupeine hydrolysate could be unequivocally identified. The preparative separation of the hydrolysis products was carried out by chromatography on columns of silicic acid–Celite (Johns Manville Co. Ltd., London, S.W 1) (Green & Kay, 1952).

DNP-DL-proline was hydrolysed with 12N hydrochloric acid in sealed tubes for 1 hr. at 100°, and after a fourfold dilution of the solution the coloured products were extracted into ethyl acetate. After washing with water to remove hydrochloric acid and after removal of the solvent *in vacuo*, there remained an orange-yellow oil from which some crystalline material separated on standing overnight. Examination of the oily residue by paper chromatography showed the presence of 2:4-dinitrophenol, a small amount of unchanged DNP-proline, and two other coloured hydrolytic products *A* and *B*. After extraction with hot benzene a solid residue was obtained, which on recrystallization from benzene yielded a lemon-yellow crystalline solid, m.p. 142–142·5°. This was shown by paper chromatography to be product *A*, but a considerable proportion of *A* remained in the first benzene extract.

In subsequent experiments the separation of *A*, *B* and 2:4-dinitrophenol from each other was effected by chromatography on silicic acid–Celite columns with acetic acid–ethyl acetate–light petroleum (Green & Kay, 1952) as the developing solvent. The separation, which was not visually apparent on a preparative scale, was followed by paper chromatography of samples from the eluate fractions. 2:4-Dinitrophenol was least strongly adsorbed and was completely separated from *A*, but in some experiments an overlap of the *A* and *B* bands was experienced, and it was found necessary to concentrate and rechromatograph the intermediate fractions containing both *A* and *B*. After removal of the solvent from the pooled fractions, *A* was obtained as a yellow oily solid and *B*, admixed with residual DNP-proline, as a yellow oil.

Product *A* was readily recrystallized from hot benzene and was obtained as a pure crystalline yellow solid, m.p. 142–142·5°, with the formula $C_{11}H_{12}O_6N_3Cl$. The chlorine was not liberated by warming with ethanolic silver nitrate, but, when an attempt was made to convert the chloro compound into a phenolic ether, halide was liberated and a small amount of DNP-proline was isolated. Some DNP-proline was formed when *A* was distilled at 170° in a high vacuum. Product *A* dissolves in aqueous sodium bicarbonate, and potentiometric titration indicates the presence of a free carboxyl group. Thus assuming that the compound retains a dinitrophenyl residue the formula may be written as

$$C_4H_7(Cl)(CO_2H).NH.C_6H_3(NO_2)_2.$$

As *A* contains one molecule of HCl more than the starting product, DNP-proline, these facts suggest that the proline ring has been broken on heating with hydrochloric acid with the formation of one of the DNP-aminochlorovaleric acids I or II.

Product *A* gave an almost theoretical yield of proline after hydrolysis with either 6N or 12N hydrochloric acid at 100° for 16 hr. Reaction with ammonia yields ornithine and an unidentified ninhydrin-positive substance, whilst hydrolysis with baryta yields proline and another unidentified ninhydrin-positive substance.

Further purification of the *B* fraction to remove DNP-proline was effected by chromatography on a powdered cellulose column with the same solvent as was used in the paper chromatographic system *B*1 (see Experimental). The hydrolytic product *B* was then crystallized by slow evaporation of the solvent from a solution in a mixture of ether and light petroleum and was obtained as a crystalline yellow solid, m.p. 107·5–109°, with the formula $C_{11}H_{12}O_6N_3Cl$. Product *B* resembles *A* in that warming with ethanolic silver nitrate liberates no halide and potentiometric titration indicates the presence of a free carboxyl group. Product *B* also yields a small amount of DNP-proline when distilled in a high vacuum.

The hydrolytic reactions of *B* are practically identical with those of *A* at 100°: hydrolysis with hydrochloric acid, both 6N and 12N, gives an 80–90 % yield of proline, hydrolysis with ammonia gives ornithine and an unidentified ninhydrin-positive substance, and hydrolysis with baryta yields proline and another unidentified ninhydrin-positive substance.

The absorption spectra of *A* and *B* were found to be very similar over the range 320–450 mμ. and to agree with the absorption spectra of open-chain DNP-amino acids such as DNP-alanine rather than DNP-proline (cf. Schroeder, Honnen & Green, 1953).

The general properties of the hydrolytic products *A* and *B* described above suggested that these two compounds were the isomeric DNP-aminochlorovaleric acids (I) and (II) and this was proved by synthesis of these compounds as shown in Figs. 1 and 2 (FDNB represents 1-fluoro-2:4-dinitrobenzene).

Initially (III) was prepared from α-DNP-δ-benzoylornithine (Sanger, 1946), but owing to the difficulty of hydrolysis of this substance we were able to obtain only a very small amount of the required product. It was, however, prepared in good yield from DL-ornithine through the δ-benzyloxycarbonyl derivative (IV).

The direct routes from (III) and (VI) to (I) both yielded mixtures of DNP products which were shown by paper chromatography to include the required product (I). By the chlorination of (V) with thionyl chloride, followed by hydrolysis of the acid chloride thus formed, α-DNP-amino-δ-chlorovaleric acid (I) was obtained as a yellow crystalline solid which was shown by mixed melting point and by paper chromatography to be identical with the hydrolysis product *A*. Although, in general, good yields of (V) were obtained from α-amino-δ-hydroxyvaleric acid, during one attempted preparation of (V) the product obtained after crystallization was the δ-lactone (VI). In like manner the action of nitrous acid on (III) gave mainly (V), as shown by paper chromatography, but after crystallization only the δ-lactone (VI) was isolated.

The product (II), obtained by interaction of δ-DNP-DL-ornithine with nitrosyl chloride, behaved identically with hydrolysis product *B*, but attempts to isolate it in crystalline form were unsuccessful. The alternative route, however, in which the α-amino group of DL-ornithine was first replaced by chlorine (Hamilton, 1952) gave α-chloro-δ-DNP-aminovaleric acid (II) in good yield (Fig. 2). The identity of *B* with (II) was established by mixed melting

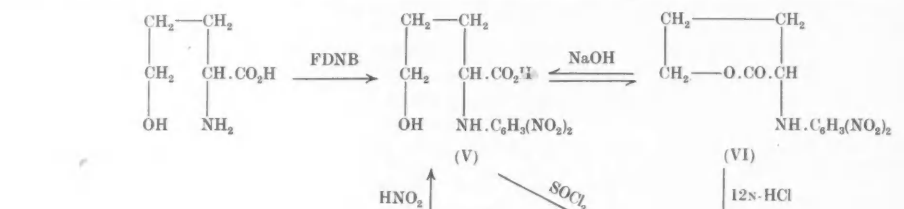


Fig. 1. Synthesis of α-DNP-amino-δ-chlorovaleric acid (I).

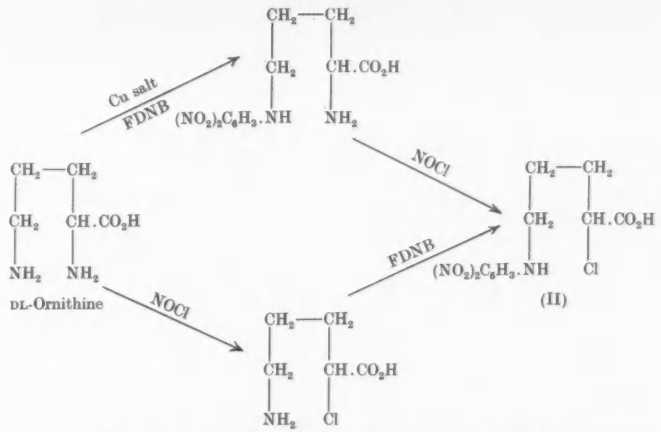
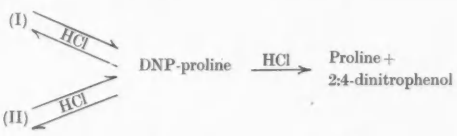


Fig. 2. Synthesis of α-chloro-δ-DNP-aminovaleric acid (II).

point and confirmed by paper and column chromatography.

This conversion of DNP-proline into the isomeric DNP-aminochlorovaleric acids represents an additional technique for the opening of the pyrrolidine ring (cf. Taylor & Baker, 1949). The reactions appear to be reversible, as both (I) and (II) yield some 5–10% of DNP-proline on heating with 12N hydrochloric acid at 100° for 1 hr.

It is suggested that the mechanism of the hydrolysis of DNP-proline with hydrochloric acid probably involves three simultaneous side reactions, two of which are reversible and result in the production of the DNP-aminochlorovaleric acids (I) and (II), whereas the third is an irreversible hydrolysis of DNP-proline to proline and 2:4-dinitrophenol, thus:



Support for this mechanism is afforded by the facts that both (I) and (II) give an almost quantitative yield of proline and considerable amounts of dinitrophenol after hydrolysis with hydrochloric acid at 100° for 16 hr. The extensive breakdown of a DNP-amino acid on acid hydrolysis into the component amino acid and dinitrophenol is not general, and according to Schramm & Braunitzer (1953) is restricted to DNP-proline, although Acher & Laurila (1953) have shown that hydrolysis of DNP-glycine and DNP-cystine also results in the formation of the corresponding amino acids. Thus the direct hydrolysis of the DNP-aminochlorovaleric acids (I) and (II) would appear unlikely, and even if it did take place it should yield the corresponding aminochlorovaleric acids. It is improbable that these acids could be intermediates in the formation of proline from DNP-proline, as it has been shown by Albertson & Fillman (1949) that α-chloro-δ-aminovaleric acid does not undergo ring-closure to form proline under acid conditions, and we have found no trace of any amino acid other than proline in the aqueous fractions of the acid hydrolysates of (I) and (II).

Our work on the breakdown of DNP-proline is not in agreement with the results of Acher & Laurila (1953), who suggest that the hydrolysis of DNP-proline with hydrochloric acid is a first-order reaction resulting in the formation of proline and dinitrophenol. An examination of the hydrolysis of DNP-proline under conditions similar to those of Acher & Laurila, namely, a concentration of 100 μg. of DNP-proline/ml. of 10N hydrochloric acid in a sealed, evacuated tube, but at 100° instead of 105°, showed a recovery of DNP-proline of less than 10% after 2 hr. instead of about 60% as found by Acher & Laurila. On the other hand, we recovered at least 50% as the mixed hydrolytic products (I) and (II), and it would therefore appear that the fractionation techniques used by Acher & Laurila did not separate DNP-proline from the breakdown products (I) and (II).

It seemed desirable to isolate the products of hydrolysis of DNP-L-proline with hydrochloric acid, as the characterization of these products could assist in a more rigid identification of *N*-terminal proline in natural compounds than can be obtained by paper and column chromatography.

Although we took such precautions as the exclusion of light and the use of anhydrous conditions

for purification (Rao & Sober, 1954), we were unable to isolate crystalline material, other than dinitrophenol, from the hydrolytic products of either DNP-L-proline or DNP-salmine. Failure to obtain DNP derivatives in crystalline form may be due to the generally lower melting points of derivatives of L-amino acids (Rao & Sober, 1954) or to partial racemization.

In the absence of crystalline hydrolytic products of DNP-L-proline, the identification of *N*-terminal proline after hydrolysis of the DNP-protein depends on identification by chromatography. As would be expected, the natural hydrolytic products from DNP-protein containing *N*-terminal proline behave in an identical manner on chromatograms to the synthetic products. In general, the following scheme, based on the reactions just described of the two DNP-aminochlorovaleric acids (I) and (II), should result in a positive identification of *N*-terminal proline in peptides or proteins.

The DNP-protein is hydrolysed at 100° with 12N hydrochloric acid for the minimum time (preferably 2–4 hr.) necessary to release most of the DNP-amino acid. The ether-soluble extract is then examined by chromatography on paper, with systems *B*1 or *A*2, whereby 5% of residual DNP-proline can be detected if the hydrolysis products are applied at a concentration equivalent to 50 μg. of original DNP-proline for each spot. If dinitrophenol is present in excessive amounts it is desirable to remove this substance, before chromatography, by high-vacuum sublimation of the ether-soluble extract at 100°.

If the presence of additional DNP-amino acids is indicated, or for further confirmation of the presence of *N*-terminal proline, the ether-soluble extract should be hydrolysed for 16 hr. at 100° with 6N hydrochloric acid. After extraction with ether, the presence of proline in the aqueous extract and of appreciable amounts of dinitrophenol in the ether extract would positively identify proline as an *N*-terminal group. On the other hand, the identification of proline but absence of dinitrophenol would indicate a DNP-peptide in which proline was not the *N*-terminal group. The remaining DNP-amino acids, if any, in the ether extract can be identified directly by paper chromatography and confirmed by alkaline hydrolysis to the corresponding amino acids.

In this manner salmine was shown to have proline as the *N*-terminal amino acid, but a clupeine component *Y* was found (Scanes & Tozer, 1956) to have both proline and alanine as *N*-terminal amino acids.

EXPERIMENTAL

All melting points are corrected. Micro-analyses for C, H, N and Cl are by F. E. Charlton, Porton, and by Weiler and Strauss, Oxford.

Table 1. *R_F values of some DNP derivatives on paper chromatograms*

For details of the solvent systems used see text.

DNP derivative	R_F value in solvent system			
	*A*1	*BE*2	*A*2	*B*1
DNP-alanine	0·25	0·73	0·23	0·30
α-DNP-amino-δ-hydroxyvaleric acid	0·19	0·79	0·17	0·42
α-DNP-amino-δ-valerolactone	0·75	0·10	0·17	0·0
DNP-arginine	0·24	0·68	0·37	0·35
DNP-glycine	0·14	0·64	0·12	0·24
α-DNP-ornithine	0·17	0·78	0·12	0·48
δ-DNP-ornithine	0·16	0·73	0·10	0·44
DNP-proline	0·25	0·76	0·22	0·42
DNP-serine	0·13	0·74	0·10	0·37
DNP-valine	0·41	0·73	0·50	0·34
Hydrolytic product *A*	0·36	0·66	0·52	0·26
Hydrolytic product *B*	0·21	0·67	0·30	0·26
2:4-Dinitrophenol	0·31	0·60	0·32	0·26

Paper chromatography. Four solvent systems, *A*1, *BE*2, *A*2 and *B*1, were used in this investigation, and by choice of a suitable combination of some or all of these systems it was possible to distinguish between any of the DNP derivatives listed in Table 1. *A*1 and *BE*2 were based on the systems of Blackburn & Lowther (1951) in which an organic solvent was equilibrated with phthalate buffer (50 ml. of 0·1M potassium hydrogen phthalate:45·45 ml. of 0·1N-NaOH), pH 6·0, and the top phase was used as developing solvent. System *A*1 consisted of *tert.*-pentanol–buffer (4:1, v/v), and *BE*2 of 1 vol. of benzyl alcohol–ethanol (9:1, v/v) and 3·4 vol. of buffer. The developing solvent of system *A*2 (Bowes & Moss, 1953) was the top phase from a mixture of equal volumes of *n*-pentanol and 2N-NH_3 soln., but the developing solvent of system *B*1 (Rovery & Fabre, 1953) was an aqueous buffer (pH 6·2) prepared from M trisodium citrate (100 ml.) and N-HCl (7·0 ml.). The paper was Whatman no. 1 filter paper and was pretreated with phthalate buffer (pH 6·0) for use with systems *A*1 and *BE*2. The DNP-derivative in ethyl methyl ketone (3 mg./ml.) was applied (5 μl.) to the paper with a micropipette, the solvent being removed in a current of air. The chromatograms were run at 20°, usually for 16 hr. with systems *A*1, *A*2 and *B*1. With system *BE*2 the paper, after application of the DNP derivatives, was equilibrated overnight in the chromatograph tank containing the *BE*2 aqueous phase before development of the chromatogram for a period of 3–4 hr.

The R_F values for the DNP compounds investigated during this work are given in Table 1. Variations in R_F values of individual DNP-amino acids were observed, especially with systems *BE*2 and *A*1, and hence control DNP-amino acids were always run with the unknown samples.

Conditions for hydrolysis of DNP-proline. The early work on hydrolytic conditions was designed to obtain a maximum recovery of DNP-proline from DNP-protamines containing proline as the *N*-terminal amino acid, but it became apparent that DNP-proline was not stable to any great extent under the conditions necessary to remove completely the DNP-prolyl group from the protein. Thus hydrolysis of DNP-DL-proline for 16 hr. with 12N-HCl

yielded less than 5% of DNP-proline and less than 10% of DNP products other than 2:4-dinitrophenol. Even under the milder conditions of hydrolysis with 96% acetic acid at 100° for 16 hr., only 50% of the original DNP-proline was recovered, whereas under these conditions DNP protein was not effectively hydrolysed.

The preliminary experiments indicated that optimum recovery of DNP compounds other than dinitrophenol was obtained by the use of 12N-HCl for hydrolysis of DNP-proline, and experiments to determine the optimum time were carried out for times varying from 0·25 to 16 hr. Some 10–20% DNP-proline was recovered after 45 min. at 100°, but only 5–10% was detected after 4 hr., by which time considerable amounts of 2:4-dinitrophenol and proline were present in the hydrolysate. For the preparative isolation of the DNP-proline hydrolytic products *A* and *B*, a hydrolysis time of 1 hr. was selected as giving the optimum yield of *A* and *B* combined with the minimum amount of residual DNP-proline. For DNP-salmine, on the other hand, a hydrolysis time of 4 hr. was preferred, as this gave a reasonable yield of hydrolytic products and the minimum of DNP-prolyl peptides.

*Isolation and purification of the products of acid hydrolysis of DNP-*DL*-proline*

DNP-DL-Proline (1 g. divided into four lots) was hydrolysed with a total of 400 ml. of 12N-HCl in sealed tubes at 100°. The contents were agitated to effect solution and heating was continued for a further 1 hr. After cooling, the bulked solution was diluted with 3 vol. of water and extracted with solvent. Ether was used in the early experiments, but subsequently ethyl acetate was preferred because of the more favourable partition of the coloured hydrolysis products between the solvent and either dilute acid or water. Two extractions with ethyl acetate (2 × 600 ml.) were carried out and the bulked extract was washed with water. The solvent was then removed *in vacuo*, leaving a light orange-yellow oil which on standing gave a small quantity of yellow crystalline solid. In one experiment the oil was separated from the solid by extraction with hot benzene (15 ml.) and the solid recrystallized from hot benzene, when pure hydrolysis product *A* (114 mg.), m.p. 142–142·5°, was obtained. In general, however, the hydrolytic products were separated by chromatography on a silicic acid–Celite column (Green & Kay, 1952).

Moist silicic acid (500 g.) (British Drug Houses Ltd.) was heated at 200° for 2 hr., cooled in a desiccator, ground and passed through an 85-mesh sieve. The yield (110–250 g.) varied unpredictably from batch to batch and some batches were very unsatisfactory for chromatography of DNP products. The acid was thoroughly shaken with one-half its weight of sieved Celite 545 and 250 g. of the mixture was slurried with acetone and poured into a tube of 58 mm. diameter. A positive pressure (8 cm. Hg) was applied, and the column, which was at least 15 cm. in height, was prewashed with dry ether (250 ml.) followed by light petroleum, b.p. 60–70° (250 ml.), and finally with the developing solution (250 ml.). The latter consisted of acetic acid (3 vol.) and ethyl acetate (12 vol.) made up to 100 vol. with light petroleum, b.p. 60–70°. The hydrolytic products, dissolved in the minimum volume (about 4 ml.) of a mixture of acetone and light petroleum b.p. 60–70° (1:1, v/v), were applied to the column and the developing solvent (4·5 l.) was added. 2:4-Dinitrophenol was eluted before the yellow bands had reached the bottom of the column. Complete separation into bands of *A* and *B*, which was observed with columns of small diameter, was not obtained with the 58 mm. diameter columns. Elution was continued until all the yellow material had been removed, the eluate being collected in 25 ml. fractions with an automatic fraction collector. Samples of each fraction, after evaporation to dryness, were examined by paper chromatography in system *A* 1, and by the use of these results the eluate fractions were combined to give pooled fractions containing *A* and *B* respectively. In a typical experiment product *A* was obtained in fractions 1–27 and *B* in fractions 36–86, the intermediate fractions containing a small overlap of *A* and *B*.

Solvent was removed from the pooled fractions by distillation *in vacuo*, the last traces of acetic acid being eliminated by redistillation of the residue *in vacuo* with added benzene (20 ml.). Product *A* was readily recrystallized from boiling benzene, and in a typical experiment 275 mg. of pure crystalline *A* was obtained.

The unchanged DNP-proline, which was present in the pooled fraction containing *B*, was separated from *B* by chromatography on a cellulose column. The mixture, dissolved in ethyl acetate, was applied to a column (58 mm. diam.) of Whatman cellulose powder and the chromatogram developed with 2 l. of the buffer (pH 6·2) used in the paper-chromatography system *B* 1. DNP-proline appeared in the early eluate fractions, and product *B* in subsequent fractions, and the separation, which was complete, was followed by paper chromatography of samples of the eluate fractions by the use of system *B* 1. The fractions containing *B* were pooled, acidified with 6N-HCl and extracted with ethyl acetate. The ethyl acetate solution was washed with water and the solvent removed by distillation *in vacuo*. Crystallization was achieved by dissolving the residue in ether (50 ml.), adding light petroleum (b.p. 60–80°) (50 ml.), and allowing the solvent to evaporate slowly in a current of dry filtered air, when yellow crystals of *B* separated (140 mg.). The product was purified by two further crystallizations, yielding yellow crystals (83 mg.) of *B*, m.p. 107·5–109°.

General properties of hydrolytic products A *and* B

Product *A* crystallized from benzene in yellow prisms, m.p. 142–142·5°. Its ultraviolet absorption spectrum in 1% $NaHCO_3$ showed maxima at 265 mμ. (ε 9700) and 361 mμ. (ε 17 800) and a minimum at 300 mμ. (ε 3100). (Found: C, 42·05; H, 3·8; N, 13·2; Cl, 11·3; equiv. by titration, 322. $C_{11}H_{12}O_6N_2Cl$ requires C, 41·6; H, 3·8; N, 13·2; Cl, 11·2%; equiv. as monobasic acid, 318.) When heated in a high vacuum at 170–180° it distilled to yield a yellow oil which was shown by paper chromatography, with systems *A* 1 and *B* 1, to consist mainly of unchanged *A* with about 20% of DNP-proline.

Product *A* is sparingly soluble in water but dissolves readily in $NaHCO_3$. Potentiometic titration in aqueous acetone solution with 0·05N-NaOH, which had been standardized under identical conditions against pure DNP-alanine, indicated the presence of a free carboxyl group. It forms an insoluble silver salt, but warming with acidified ethanolic $AgNO_3$ does not liberate halide. On heating with phenol and 2N-NaOH at 100° for 1 hr., halide was liberated and alkali-soluble material was obtained which gave a solid on acidification. Recrystallization of the latter from

a mixture of ether and light petroleum gave yellow crystals of impure DNP-proline, m.p. 175–177°, not depressed on admixture with authentic DNP-DL-proline. Although some *A* was recovered after heating with 12N-HCl at 100° for 16 hr., a quantitative yield of proline was obtained after heating with 6N-HCl for 16 hr. Ornithine and unidentified ninhydrin-positive material were obtained after heating with aqueous NH_3 (sp.gr. 0·88) at 100° for 4 hr., and proline and unidentified ninhydrin-positive material were obtained after heating with saturated $Ba(OH)_2$ at 100° for 4 hr.

Compound *B* was obtained in the form of yellow prisms, m.p. 107·5–109°. Its ultraviolet absorption spectrum in 1% $NaHCO_3$ showed maxima at 265 mμ. (ϵ 8400) and 362 mμ. (ϵ 18 000) and a minimum at 300 mμ. (ϵ 2100). (Found: C, 41·2; H, 3·9; N, 13·1; Cl, 11·1; equiv. by titration, 314. $C_{11}H_{12}O_6N_3Cl$ requires C, 41·6; H, 3·8; N, 13·2; Cl, 11·2%; equiv. as monobasic acid 318.)

Like product *A*, product *B* yields some DNP-proline on heating in high vacuum and no halide when heated for a short time with acidified ethanolic $AgNO_3$. The presence of a free carboxyl group was shown by potentiometric titration. Product *B* also yielded products similar to *A* after hydrolysis, namely, proline (in high yield) after 16 hr. with 6N- and 12N-HCl at 100°, ornithine and unidentified ninhydrin-positive material after 4 hr. with aqueous NH_3 (sp.gr. 0·88) at 100°, and proline and unidentified ninhydrin-positive material after 4 hr. with saturated $(BaOH)_2$ solution at 100°.

Chromatographic behaviour of A *and* B. In system *A* 1, product *A* is well separated from *B* and from DNP-proline, but *B* is not completely separated from DNP-proline. Systems *B* 1 and *BE* 2, in which *A* and *B* run together, are of value in separating DNP-proline from these products. In system *A* 2, 2:4-dinitrophenol and *B* run together but DNP-proline and *A* are well separated, and as DNP-proline runs slowly this system is well suited for the detection of relatively small amounts (1–2 μg.) of DNP-proline in the presence of the breakdown products. Excessive amounts of dinitrophenol may cause interference, and should be removed by high-vacuum sublimation at 100°.

Syntheses

α-2:4-*Dinitrophenyl-δ-benzyloxycarbonyl-*DL-*ornithine.* δ-Benzyloxycarbonyl-DL-ornithine (IV) was obtained by slight modifications of the method used by Synge (1948) for the preparation of δ-benzyloxycarbonyl-L-ornithine. The reaction product (IV) from DL-ornithine hydrobromide (1·07 g.) on recrystallization from water gave colourless plates (0·8 g.), m.p. 251–252°; Harris & Work (1950) report m.p. 256°.

δ-Benzyloxycarbonylornithine (IV) (0·53 g.) was mixed with $NaHCO_3$ (0·57 g.) in water (8 ml.), a solution of FDNB (0·57 ml.) in ethanol (16 ml.) added and the mixture shaken for 2 hr. The ethanol was distilled *in vacuo* and the residue diluted with water (10 ml.) and extracted with ether (2 × 20 ml.). A small amount of insoluble material was removed from the aqueous solution by filtration and the DNP derivative precipitated as an oil by the addition of 6N-HCl (4 ml.). As the oil did not solidify on standing at 0° it was extracted with ether (25 ml.) and after washing with 0·5N-HCl and with water the ethereal solution was dried (Na_2SO_4) overnight. The ether was removed *in vacuo*, considerable frothing taking place towards the end of the distillation. The amorphous yellow solid (0·8 g.) was used directly for the next stage.

α-2:4-*Dinitrophenylornithine* (*III*). The crude α-2:4-dinitrophenyl-δ-benzyloxycarbonylornithine (0·3 g.) was heated at 60–70° with 12N-HCl (5·5 ml.) for 1 hr. After removal of HCl *in vacuo* the residue was dissolved in water (20 ml.) and extracted with ether, but no trace of ether-soluble starting material was observed. The aqueous solution was evaporated to dryness *in vacuo*, and after addition of water the evaporation was repeated several times. The residue was dissolved in boiling water (30 ml.), filtered and neutralized with pyridine (0·1 ml.). On standing, yellow needles (160 mg.) separated, m.p. 220–221° (decomp.). The mixed m.p. with an authentic sample of α-2:4-dinitrophenylornithine (m.p. 220–222°), prepared according to the method of Sanger (1946), was not depressed.

Reactions of α-2:4-*dinitrophenylornithine with* NOCl *and* HNO_2. A solution of NOCl, prepared by grinding $AgNO_2$ (0·5 g.) with ice-cold 6N-HCl (5 ml.), was added to α-2:4-dinitrophenylornithine (50 mg.) in peroxide-free dioxan (5 ml.), and the clear solution heated at 65°. Heating was continued for 5 hr., by which time some coloured ether-extractable material had been formed. The solution was cooled, diluted with water (5 ml.) and extracted with ether (3 × 30 ml.). The ether extract was washed with water (30 ml.) and the ether removed *in vacuo*. The oily residue, which could not be crystallized, was found by paper chromatography to contain five yellow-coloured components, one of which corresponded with the required δ-chloro derivative (I).

Because of the difficulty in replacing the δ-amino group directly by Cl, the action of nitrous acid was investigated, and under the normal analytical conditions an almost quantitative release of N_2 was obtained.

α-2:4-Dinitrophenylornithine (19 mg.) in acetic acid (1 ml.) was shaken with saturated $NaNO_2$ until evolution of gas had ceased (1 hr.). The solution was distilled to dryness *in vacuo* and the residue extracted with a mixture of ethyl acetate (10 ml.) and water (10 ml.). The ethyl acetate solution, which contained all the yellow-coloured material, was distilled *in vacuo*. Paper chromatography of the solid residue (with system *A* 1) showed 2 spots, the stronger one corresponding to α-2:4-dinitrophenylamino-δ-hydroxyvaleric acid (V). Crystallization from a mixture of ethyl acetate and light petroleum (b.p. 40–60°) gave yellow crystals, m.p. 179–181° (decomp.), not depressed on admixture with α-2:4-dinitrophenylamino-δ-valerolactone (VI) (see below).

α-2:4-*Dinitrophenylamino-δ-hydroxyvaleric acid* (*V*). α-Amino-δ-hydroxyvaleric acid (0·8 g.), prepared from γ-butyrolactone by the method of Plieninger (1950), was mixed with $NaHCO_3$ (1·06 g.) in water (20 ml.). A solution of FDNB (1·22 g.) in ethanol (40 ml.) was then added and the mixture shaken for 2 hr. The ethanol was removed *in vacuo* and the residue diluted with water (40 ml.) and extracted with ether (2 × 40 ml.). The aqueous solution was acidified with 2N-H_2SO_4 (4·5 ml.), stood at 0–5° (1 hr.), and the precipitate filtered, washed with cold water (10 ml.) and dried. Crystallization of the crude material (1·6 g.) from a mixture of ethyl acetate and light petroleum, b.p. 60–80°, gave pure α-2:4-*dinitrophenylamino-δ-hydroxyvaleric acid* as yellow prisms, m.p. 141·5–142°. (Found: C, 44·2; H, 4·0; N, 13·6. $C_{11}H_{13}O_7N_3$ requires C, 44·1; H, 4·35; N, 14·0%.)

α-2:4-*Dinitrophenylamino-δ-valerolactone* (*VI*). In one preparation of the δ-hydroxyvaleric acid (V) (see above) the

material separated as an oil on attempted crystallization from a mixture of ethyl acetate and light petroleum. After removal of the solvent *in vacuo* the residue was recrystallized from a mixture of ethyl acetate and light petroleum and gave pure α-2:4-*dinitrophenylamino*-δ-*valerolactone* as yellow prisms (1·3 g.), m.p. 186–188°. (Found: C, 47·0; H, 3·95; N, 15·0. $C_{11}H_{11}O_6N_3$ requires C, 47·0; H, 3·9; N, 14·95%.)

The lactone (VI), which was insoluble in Na_2CO_3, was partially reconverted into the δ-hydroxy acid (V) by shaking an ethyl acetate solution with 0·1 N-NaOH; identification of the δ-hydroxy acid was made by mixed m.p. and by paper chromatography, with systems *A* 1, *BE* 2 and *B* 1.

α-2:4-*Dinitrophenylamino*-δ-*chlorovaleric acid* (*I*). α-2:4-Dinitrophenylamino-δ-hydroxyvaleric acid (130 mg.) in pyridine (0·1 ml.) was cooled in an ice bath and $SOCl_2$ (0·15 ml.) added from a micropipette during 10 min. After heating at 50° for 90 min. the reaction mixture was cooled in an ice bath, ice (3 g.) was added, and the mixture of oil and solid was stirred while it warmed to room temp. After acidification with 6 N-HCl (1·5 ml.) the dark-red solution was extracted with ethyl acetate (2 × 5 ml.). The ethyl acetate solution was washed with 2 N-HCl (2 × 5 ml.) and with water (2 × 10 ml.) and extracted with 1% Na_2CO_3 (2 × 10 ml.), when much of the red colour remained in the ethyl acetate. The Na_2CO_3 extract was washed with ethyl acetate (2 × 10 ml.), acidified with 2 N-HCl (10 ml.), and re-extracted with ethyl acetate (2 × 10 ml.). The ethyl acetate extract was washed with 2 N-HCl (10 ml.) and water (2 × 10 ml.) and dried (Na_2SO_4) overnight. After removal of solvent *in vacuo* the residue was shown by paper chromatography (with systems *A* 1, *A* 2 and *B* 1) to be the required acid (I) and to be almost free of other DNP compounds. In a preliminary experiment, where the reaction mixture was not cooled during the $SOCl_2$ addition, the reaction product was shown to contain DNP-proline as well as the required acid (I). Crystallization of the residue from benzene gave a crystalline solid (21 mg.) which on recrystallization gave α-2:4-dinitrophenylamino-δ-chlorovaleric acid (I) as yellow prisms, m.p. 140·5–141·5°, not depressed on admixture with the acid hydrolysis product *A* from DNP-DL-proline.

Alternatively, the chlorination of the δ-hydroxy acid (V) was effected with PCl_5 in acetyl chloride at 0°, the product being decomposed with ice and ether extracted from acid solution. Examination by paper chromatography in systems *A* 1, *A* 2 and *B* 1 showed the presence of the required chloro compound (I) and of DNP-proline, but the products were not isolated as crystalline solids.

α-*Chloro*-δ-2:4-*dinitrophenylaminovaleric acid* (*II*). α-Chloro-δ-aminovaleric acid (100 mg.), prepared from DL-ornithine by the method of Hamilton (1952), was mixed with $NaHCO_3$ (140 mg.) in water (2 ml.). A solution of FDNB (190 mg.) in ethanol (4 ml.) was then added and the mixture shaken for 2 hr. The ethanol was removed *in vacuo* and the residue diluted with water (4 ml.) and extracted with ether (2 × 5 ml.). The aqueous solution was acidified with 2 N-HCl (1 ml.) and stood at 0° for 48 hr. The yellow product, which separated as an oil, was extracted into ether (2 × 15 ml.) and after drying (Na_2SO_4) the ether was removed *in vacuo*. The residual oil solidified after standing in the dark for some days at 20°. It was crystallized by solution in hot ethyl acetate followed by careful addition of light petroleum, b.p. 40–60°, until a slight turbidity was produced. This was allowed to clear before re-warming and addition of more light petroleum. After standing overnight the mixture gave pure α-*chloro*-δ-2:4-*dinitrophenylaminovaleric acid* (*II*) as yellow prisms, m.p. 106–108°, not depressed on admixture with product *B* obtained by hydrolysis of DNP-DL-proline with HCl. (Found: C, 41·9; H, 4·1; N, 13·1. $C_{11}H_{12}O_6N_3Cl$ requires C, 41·6; H, 3·8; N, 13·2%.)

SUMMARY

1. The stability of DNP-proline has been examined under conditions of hydrolysis with hydrochloric acid such as are used in the identification of *N*-terminal residues of proteins.

2. Two hitherto undescribed hydrolytic products of DNP-DL-proline have been isolated and details of their general properties are given.

3. These products have been shown by synthesis to be α-2:4-dinitrophenylamino-δ-chlorovaleric acid and α-chloro-δ-2:4-dinitrophenylaminovaleric acid.

4. A scheme for the identification of *N*-terminal proline is suggested in the light of these findings.

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Metabolism of Bladder Carcinogens

2. THE METABOLIC PATH OF 2-[8-^{14}C]NAPHTHYLAMINE IN THE RABBIT AND IN THE RAT*

By A. R. SOMERVILLE, A. F. HENSON, M. ELISABETH COOKE, MURIEL E. FARQUHARSON† and M. W. GOLDBLATT
Akers Research Laboratories and Industrial Hygiene Research Laboratories, Imperial Chemical Industries, Ltd., The Frythe, Welwyn, Herts

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In a previous paper (Henson, Somerville, Farquharson & Goldblatt, 1954) we reported the results of an investigation into the distribution of 2-[8-^{14}C]naphthylamine or its metabolites in the rat, following intraperitoneal injection. We now present the results of a similar study on rabbits, together with some additional results on rats.

MATERIALS AND METHODS

These were, with minor modifications, as previously described. Rabbits weighed from 1 to 2·7 kg.

Measurement of ^{14}C. The correction for the dead time of a Geiger counter capacitatively coupled to a square-pulse-quenching unit proposed by Wyard (1953) has been found to give a slightly better agreement at high counting rates than the correction previously used (Henson, 1953). Recalculation on this basis reduced the measured efficiency of the counters from 72·1 to 70·9 % and the activity of the naphthylamine as assayed by the wet-oxidation method from $2{\cdot}751 \pm 0{\cdot}014 \times 10^7$ to $2{\cdot}719 \pm 0{\cdot}013 \times 10^7$ counts/min./mg.

Oxidation techniques. The method of preparing CO_2 from animal tissues for measurement in Geiger counters, which had been developed with rats, was unsatisfactory with most rabbit tissues. An impurity in the gas spoiled the counter characteristics, giving a slow rise to a plateau of high slope. This difficulty has been met by other workers using the method; it is apparently due to the production from protein of oxides of nitrogen which can be removed by exposing the gas to hot reduced copper (Bradley, Holloway & McFarlane, 1954; Glascock, 1954). We have introduced this step into our routine preparation of CO_2 from rabbit tissues and usually obtain satisfactory plateaux. We have not encountered this difficulty with any rat tissues, but almost all counter fillings from rabbit tissues have been unsatisfactory without the extra purification.

Anaesthesia. For the experiments in which bile was collected from cannulae inserted in the common bile duct, anaesthesia was initially produced by intraperitoneal injection of Nembutal (50 mg./kg. of body wt.) and was maintained throughout the experiment with *cyclo*propane–oxygen in rabbits and ether in rats. *cyclo*Propane is liable to produce respiratory depression and sudden failure in rabbits, and hence the adjustment of the ratio of *cyclo*propane to oxygen requires careful attention. The change from quiet even breathing to shallow, rapid and Cheyne-Stokes type is often very sudden.

RESULTS

Activity in the blood of rabbits after intraperitoneal injection of 2-[8-^{14}C]*naphthylamine.* The level of activity in the blood during the first few hours after injection was measured and the results for three normal rabbits (1, 2 and 3i), and for one with its bile duct ligated (7), are shown in Fig. 1. Samples of blood withdrawn from the ear were dried and weighed. The total activity in the animal's blood was calculated on the assumption that the blood lost 80 % of its weight on drying and that the weight of whole blood was 5 % of the body weight.

Partition of activity between blood plasma and blood cells in rabbits and rats. Four rabbits were injected intraperitoneally with approximately 1 mg. of 2-[8-^{14}C]naphthylamine. Blood withdrawn at

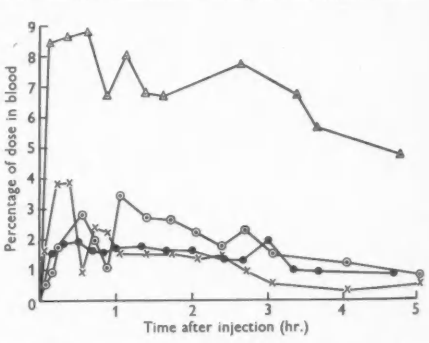

Fig. 1. Activity in blood of rabbits after intraperitoneal injection of approx. 1 mg. of 2-[8-^{14}C]naphthylamine. ●, ⊙, ×, Normal rabbits 1, 2, 3i; △, rabbit 7 with ligated bile duct.

* Part 1: Henson, Somerville, Farquharson & Goldblatt (1954).

† Present address: T. and H. Smith Ltd., Edinburgh.

various times from the ear was centrifuged and samples of plasma and cells (unwashed) were dried and weighed. The activities are shown in Table 1.

Washing the cells obtained 26 hr. after injection of the dose with four equal volumes of isotonic citrate removed 3·3, 2·2 and 2%, respectively, of the activity, but blood cells obtained from three rabbits 2 hr. after injection contained more loosely bound material, for 30–40% of the activity was removed in one citrate wash. Results for six rats are shown in Table 2. When rats 22 and 26 were killed the activity of the marrow from the femur of rat 22 was 0·3 count/min./mg. and of rat 26, none. Corresponding values for activity in blood cells were 13·6 and 10·0 counts/min./mg. The high level maintained in the blood cells of the rat is therefore not due to the continual release of new labelled cells from the marrow.

Excretion of radioactivity from rabbits after intraperitoneal injection of 2-[8-^{14}C]*naphthylamine.* The percentage of the dose excreted in the urine and faeces in three experiments is shown in Table 3. In the second experiment with rabbit 3ii a collar was fitted to prevent refection of faeces in order to provide a better comparison with the results in rats.

Table 4 shows the distribution of the radioactive material in the excreta and various organs at different times after administration for eight rabbits, in three of which the bile ducts had been previously ligated. The effect of ligating the bile duct is much less noticeable than in rats. The reduction of the amount in the gastro-intestinal tract and faeces is not so marked, and it appears

Table 1. *Distribution of activity between blood plasma and cells in rabbits after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Results are expressed as counts/min./mg. dry wt. Doses: rabbits 8 and 9, $2{\cdot}898 \times 10^7$, rabbit 10, $3{\cdot}059 \times 10^7$, rabbit 11, $2{\cdot}402 \times 10^7$ counts/min. Sample weights were such that the minimum detectable counting rate of 3 counts/min. above background was given by a sample having an activity of 0·2–0·5 count/min./mg. Samples having counting rates below 3 counts/min. are reported as zero.

Time after injection (hr.)	Rabbit 10		Rabbit 11	
	Plasma	Cells	Plasma	Cells
0·67	179·8	8·2	—	—
1·00	—	—	259·0	5·9
5·00	21·7	2·5	24·0	1·7
22·00	—	—	2·0	1·1
26·00	2·5	2·7	2·2	1·0
Time after injection (days)	**Rabbit 8**		**Rabbit 9**	
	Plasma	Cells	Plasma	Cells
1	5·6	0·5	6·2	0·4
4	7·2	0	2·3	0·6
7	2·7	0	1·9	0·4
11	1·9	0·3	0·9	0·5
18	4·9	0·4	2·8	1·1
25	0	0·2	0	0·7
33	0	0	0·6	0
40	1·0	0·2	0	0·3
42	0·3	0	—	—
47	—	—	7·5	1·3
54	—	—	1·7	0·4
61	—	—	0·5	0·2
67	—	—	0	0
69	—	—	0	0

Table 2. *Concentration in blood plasma and cells of rats after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Results are expressed as counts/min./mg. dry wt. Dose to each rat $3{\cdot}037 \times 10^7$ counts/min. Last entries pertain to blood taken at death, those in parentheses to washed cells.

Time (days)	Rat 22		Rat 23		Rat 24		Rat 25		Rat 26		Rat 27	
	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells	Plasma	Cells
1	45·3	56·4	36·6	88·3	51·8	65·2	43·7	88·8	51·7	50·5	45·6	88·3
4	12·5	45·6	8·9	75·0	6·7	60·0	9·2	68·4	8·3	44·9	10·1	69·1
7	48·4	41·5	7·6	62·0	8·6	48·0	41·6	54·9	3·4	38·3	1·6	48·7
11	1·4	32·3	8·7	40·2	3·0	37·1	3·9	42·0	2·1	32·7	2·0	33·1
14	—	28·5	2·4	20·8	3·2	29·9	0·6	25·8	1·0	40·4	—	15·9
19	—	—	2·7	12·8	9·7	18·9	0	18·3	1·6	19·0	1·0	19·1
25	—	—	2·0	7·3	0	12·8	0·7	10·3	—	—	0	9·2
26	0·5	13·6 (12·5)	—	—	—	—	—	—	—	—	—	—
28	—	—	0	5·4	0	11·5	0	8·1	0·4	10·0	0	6·6
35	—	—	0	2·8	0	6·9	0	3·5	—	—	0	4·8
42	—	—	0	2·9	0	1·5	0	1·3 (1·2)	—	—	0	2·2
47	—	—	0	0·6	—	—	—	—	—	—	0	1·4
48	—	—	—	—	0·4	1·0 (0·9)	—	—	—	—	—	—
53	—	—	0·5	0·24 (0·18)	—	—	—	—	—	—	—	—
56	—	—	—	—	—	—	—	—	—	—	0	0·4 (0·4)

that a substantial fraction of the dose reaching the gastro-intestinal tract does so by other routes than the bile. In order to investigate this the bile ducts of three rabbits were cannulated and bile was collected continuously after intraperitoneal injection of naphthylamine. The rates at which rabbits 17 and 20 secreted bile, 46·2 ml. in 5·5 hr. and 47·4 ml. in 4 hr. respectively, were of the same order as those observed by Archdeacon, Danforth & Dummit (1954). For rabbit 19 the rate was 9·8 ml. in 4·5 hr. Results are summarized in Table 5 and Figs. 2 and 3. The ratio of the concentration of activity in the bile to that in the blood (values taken from the smoothed curves in Fig. 3) decreased from 7·7 at 30 min. to 2·8 at 3·5 hr. in rabbit 19, but in rabbit 20 the ratio rose from 17·8 to 32·1 at the same times. These experiments confirm those results obtained by ligation of the bile duct in that the activity contained in the bile does not represent the great majority of that which reaches the gastro-intestinal tract, as it does in the rat. Comparable figures for a rat with a cannulated bile duct are shown in Table 5.

The bile from rabbit 19 was filtered through a semi-permeable cellulose membrane under reduced pressure. The residue on the filter was suspended in water and again filtered. The filtrates contained all the radioactivity of the original bile and gave no biuret reaction for protein, from which it is concluded that the metabolites are not at this stage firmly bound to protein.

Table 3. *Percentage of dose excreted in urine and faeces of rabbits after intraperitoneal injection of* 2-[8-^{14}C]*naphthylamine*

Dose approx. 1 mg. Rabbit 3ii was fitted with a collar.

Rabbit no.	Time after injection (days)	Urine (% of dose)	Faeces (% of dose)
1	1·3	81·7	7·8
3i	18	89·9	7·4
3ii	17	82·4	13·3

Table 4. *Percentage of dose present in tissues and excreta of rabbits after intraperitoneal injection of approx.* 1 *mg. of* 2-[8-^{14}C]*naphthylamine*

Time after injection	1 hr.	2 hr.				26 hr.		
Animal no.	6	4	5	12*	14*	10	11	7*
Urine	39·5	62·4	55·9	55·1	40·0	77·4	82·6	90·2
Faeces	—	0·003	0·3	—	—	0·6	8·7	0·03
Stomach	2·3	1·8	3·3	2·4	4·6	2·6	0·6	1·1
Small intestine	21·7	4·0	2·0	3·3	3·8	0·4	0·2	1·9
Large intestine	2·1	11·7	17·1	12·3	2·6	4·7	7·3	1·1
(Gastro-intestinal tract and faeces)	(26·1)	(17·5)	(22·7)	(18·0)	(11·0)	(8·3)	(16·8)	(4·1)
Liver	1·6	1·4	1·7	1·9	2·5	0·2	0·2	0·8
Kidneys	4·2	1·6	2·0	1·1	5·7	0·03	0·06	0·9
Carcass	—	—	—	—	21·7	1	—	4·0
Total	71·4†	82·9†	82·3†	76·1†	81·3‡	86·9	99·6†	100·0

* Bile duct previously ligated. † Excluding carcass. ‡ Includes bile 0·4.

Table 5. *Percentage of dose present in tissues and excreta of rabbits and a rat with cannulated bile ducts after injection of* 2-[8-^{14}C]*naphthylamine*

Anaesthesia was maintained throughout the experiment. Rabbit 19 was starved for 16 hr. before the experiment. Dose approx. 1 mg.

	Rabbits			Rat
Time after injection (hr.) ...	3·25	4·5	4·0	1·75
Animal no.	17	19	20	29
Bile collected	8·3	3·5	17·5	34·4
Bile in gall bladder	—	1·5	0·5	—
Gastro-intestinal tract and contents	4·4	7·8	4·0	11·0
Urine	42·0*	23·2	58·8	11·8
Bladder	1·25	0·5	2·6	0·2
Kidneys	7·2	32·9	2·3	5·2
Liver	2·4	2·8	0·7	5·1
Carcass	19·5	17·6	5·6	27·2
Recovery	85·0	89·8	92·0	94·9

* Some urine was lost.

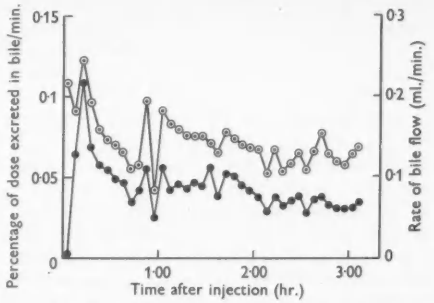

Fig. 2. Flow of bile and excretion of dose in bile of rabbit 17 after intraperitoneal injection of approx. 1 mg. of 2-[8-^{14}C]naphthylamine. ⊙, Rate of bile flow; ●, rate of excretion of dose in bile.

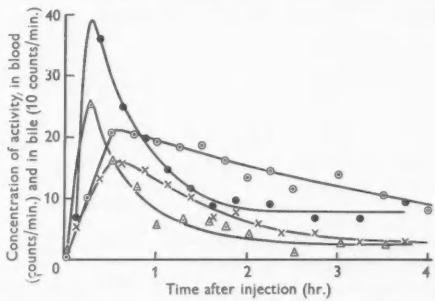

Fig. 3. Concentration of activity in blood and bile of rabbits after intraperitoneal injection of 2-[8-^{14}C]naphthylamine. Rabbit 19: dose $2{\cdot}156 \times 10^7$ counts/min.; ⊙, blood; ×, bile. Rabbit 20: dose $3{\cdot}765 \times 10^7$ counts/min.; △, blood; ●, bile. The points on the bile curves represent the mean concentration in bile collected over periods of 15 or 30 min.

DISCUSSION

The variation in concentration of activity in the blood of normal rabbits follows a similar course to that in rats, rising to a maximum in 0·5–1 hr. (Fig. 1, rabbits 1, 2 and 3i), but the maximum is not so well defined and the rate of fall is lower. The effect of ligating the bile duct (rabbit 7) is less than in the rat, for apart from rising to a somewhat higher level the blood concentration follows the normal pattern, whereas in the rat the maximum is broadened and the fall delayed (Henson *et al.* 1954, Table 1). This is a reflexion of the fact that in the rabbit only a small fraction of the dose is excreted in the bile.

Table 1 shows that in the first few hours most of the activity in the blood is in the plasma. After one day the plasma concentration (on a dry-weight basis) still tends to remain higher than that in the cells, both then falling to below the level of detectability over a period of several weeks with the plasma concentration remaining the higher. This is very different from the findings with the rat (Henson *et al.* 1954, Table 3), where after periods of 10 and 15 days the activity is almost entirely in the blood cells. The further results on rats reported in Table 2 confirm this difference between the two species. After one day all six rats have comparable dry-weight concentrations in plasma and cells, but that in the plasma then decreases rapidly. The cell activity decreases much more slowly so that after 11 days the blood activity is almost wholly in the cells. Washing the cells removes very little activity. The decline of activity in the cells can be reasonably approximated by exponential curves having time constants of 17·6, 9·0, 14·5, 10·3, 16·5, 10·4 days for rats 22 to 27 respectively. If the cells were initially labelled with active material which remained fixed until the cell reached the end of its life and was then removed from the blood, a linear decrease would be expected. The average life of red cells in a rat has been reported to be 45–50 days (Burwell, Brickley & Finch, 1953); this would require a slower decrease than we have observed. The exponential decrease probably means that the activity is bound to a cell constituent which is in dynamic equilibrium, the time constant of the exponential representing the mean life of this constituent. We have as yet no explanation of the sudden rise in activity in the plasma of rats 22 and 25 at 7 days and rat 24 at 19 days. The effect in rabbit 9 at 47 days is presumably due to the same cause.

Rabbits 8 and 9 were killed 42 days and 69 days respectively after injection when the activity in the blood had reached the limit of detectability. At this stage the activities in the kidneys, liver and bone of rabbit 8 were just on the limit of detectability with none detectable in the bladder and bone marrow. There was no detectable activity in any organ of rabbit 9. Thus in the rabbit, as in the rat, naphthylamine or its metabolite is retained in the blood at least as long as in any other part of the body.

The initial excretion of the active material in the urine is much more rapid in rabbits than in rats. The former excrete over 50% of the dose in 2 hr. (Table 4), and the latter about 22% (Henson *et al.* 1954, Table 5). The total fraction excreted in the urine is also higher, over 80% compared with 65% in the rat (Table 3, and Henson *et al.* 1954, Fig. 1) and the amount reaching the gut is correspondingly less. The figures in Table 4 for the total in the faeces and gastro-intestinal tract show for normal animals a general decline with time which indicates a reabsorption of the material from the gut, as in the rat.

The proportion of the dose excreted in the bile of the rabbit is small, as shown by the slight effect of ligating the bile duct on the contents of the gastro-intestinal tract and faeces (Table 4), and it appears that a comparable amount reaches the gut by other routes. In the rat a much larger percentage reaches the gut, the great majority being carried in the bile. These conclusions were confirmed by the experiments with cannulated animals (Table 5), which show that in rabbits active material still reached the gut from extra-biliary sources in amounts generally of the same order as that excreted in the bile. In the rat 11% of the dose reached the gut in 1·75 hr. but over three times as much was found in the bile.

Naphthylamine or its metabolites appear very rapidly in the bile (Fig. 2), the rate of excretion reaching a maximum within 15 min. in rabbit 17. Apart from a somewhat sharper maximum the curve closely resembles those showing variation of concentration in the blood (Figs. 1 and 3). Within the accuracy of our measurements the concentration in the bile does not lag behind that in the blood. The transfer of the material from the blood to the bile is not merely a matter of diffusion through a membrane, for the concentration in the bile is much higher than in the blood, strikingly so in the case of rabbit 20, despite the fact that the actual volume of bile excreted by this animal was very high.

SUMMARY

1. Rabbits injected intraperitoneally with 2-[8-^{14}C]naphthylamine (approx. 1 mg.) retain radioactivity in the blood for several weeks. This activity is shared between the plasma and cells. In rats, activity is detectable in the blood for about the same length of time, but after 11 days it is entirely in the cells.

2. In one day after intraperitoneal injection of approximately 1 mg. of 2-[8-^{14}C]naphthylamine in rabbits about 90% of the dose is excreted in the urine and faeces. The initial excretion in the urine is very rapid (over 50% of the dose in 2 hr., compared with 22% for rats). Total urinary excretion in the rabbit is about 80%.

3. Excretion of radioactive material into the gut of rabbits, both by the biliary route and otherwise, is demonstrated. The total amount reaching the gut is much less than in rats, mainly because excretion in the bile is much lower. Reabsorption of active material from the gut is shown to occur.

4. The concentration of radioactivity in the bile and the blood of rabbits varies in a closely similar manner. The actual concentration in the bile at a given time is considerably higher than in the blood and may exceed it from 3 to over 30 times in anaesthetized rabbits with cannulated common bile ducts.

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The Enzymic Synthesis of Steroid Sulphates

By A. B. ROY

Department of Biochemistry, University of Edinburgh

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Soluble enzyme preparations from rat liver have been known for some time to be capable of synthesizing aryl sulphates from the appropriate phenol and sulphate ions in the presence of adenosine triphosphate (Bernstein & McGilvery, 1952*a*; DeMeio, 1952). There had been no investigation of the synthesis of steroid sulphates by such enzymes until very recently, when DeMeio & Lewycka (1955) were able to demonstrate the probable synthesis thereby of dehydro*epi*androsterone sulphate (DHAS), although the product was not characterized. The synthesis of such steroid sulphates is of particular interest as these compounds must play a part in the metabolism of steroids, and their synthesis is probably more representative of the true function of the enzyme system involved than is the commonly studied formation of the sulphates of toxic phenols. Furthermore, the molluscan enzyme hydrolysing these steroid sulphates, steroid sulphatase, showed

a high degree of specificity (Roy, 1955), and it was of interest to investigate the corresponding specificity of the more widely distributed synthetic system. The method of assay used in the present investigation allowed the ready investigation of a large number of different substrates and so was of more general application than the previously available methods for the assay of sulphate ester synthesis.

EXPERIMENTAL

Preparation of the enzyme. This was prepared by the method of Bernstein & McGilvery (1952*a*). Portions of rat liver were homogenized in a Potter & Elvehjem (1936) homogenizer with 3–4 vol. of 0·15 M-KCl containing 0·001 M ethylenediaminetetraacetic acid, pH 7. The temperature at this and all subsequent stages was kept at 0°. The homogenate was centrifuged for 2 hr. at 20 000 *g* and the supernatant, containing the enzyme, separated. The enzyme was then purified by fractional precipitation with ammonium sulphate, and the fraction precipitated between 1·7 M- and 2·3 M-$(NH_4)_2SO_4$ contained the bulk of the enzyme. This fraction was dissolved in water (0·7 ml./g. of liver) and the solution stored at −20°, at which temperature it was stable for at least 4 weeks. For assay by the method described below, preparations from male and female animals were diluted with 1 and 3 vol. of water respectively.

Assay of steroid sulphate synthesis. The following method was used in the assay of DHAS synthesis, but it was directly applicable to the assay of the synthesis of any steroid sulphate.

Buffered adenosine triphosphate (ATP) was prepared immediately before use by mixing equal vol. of 0·3 M-KH_2PO_4 (pH 6·8), 0·03 M-K_2SO_4, 0·005 M-$MgCl_2$, and 0·33 vol. of 0·04 M ATP, pH 6·8. The ATP was the disodium salt (L. Light and Co., Colnbrook, Bucks). To 0·5 ml. of buffered ATP was added 0·1 ml. of 0·4 mM dehydro*epi*androsterone (DHA) in propylene glycol followed by 0·4 ml. of enzyme solution. The final concentrations of ATP and DHA were therefore 2·0 and 0·04 mM respectively. The mixture was incubated for 2 hr. at 37° and the reaction then stopped by the addition of 5 ml. of ethanol. After standing for 15 min. the tubes were capped, and the precipitated protein was removed by centrifuging. A sample of the clear supernatant (5 ml.) was taken for the assay of DHAS as described below.

Blank determinations were simultaneously carried out, in which the enzyme was incubated separately from the remainder of the reaction mixture and added after the ethanol. Assays and blanks were always performed in duplicate.

Determination of DHAS. DHAS was assayed by a micromodification of the method already described by Roy (1956), which makes use of the solubility in chloroform of the complexes formed between methylene blue and sulphuric acid esters. This method can be applied to the determination of any steroid sulphate ester (Roy, 1956). The methylene-blue reagent used in the present method was prepared from that previously used (Roy, 1956) by diluting with an equal volume of water.

The 5 ml. sample of the protein-free solution obtained above was concentrated in a boiling-water bath for 20 min. This did not give a completely dry residue but left about 0·1 ml. of a viscous liquid, presumably propylene glycol. After cooling, the residue was taken up in 2 ml. of methylene-blue reagent, 5 ml. of chloroform was added and the mixture shaken vigorously for 20 sec. The mixture was centrifuged, the aqueous layer removed and a sample of the chloroform layer taken into a test tube and dried with Na_2SO_4. The intensity of the resulting blue solution was read in the Spekker absorptiometer with micro cells (1 cm. light path) and Ilford filter no. 608 (maximum transmission 700 mμ.) against the appropriate blank.

A calibration curve for the above method was prepared by dissolving known amounts of DHAS in a medium of composition identical with that of the assays, except that water was added in place of enzyme, followed by ethanol precipitation, etc., as described above. It is essential that the blank and calibration tubes have a composition identical with that of the assays as variations in their composition may cause variations in the shape of the calibration curve, which is not strictly linear. Ethanol precipitation minimizes these effects by the removal of the bulk of the inorganic ions which may interfere.

The recovery of known amounts of DHAS added to the incubation mixture was satisfactory, the mean recovery in the range 5–25 μg. of DHAS being 95% from solutions containing 8 mg. of protein/ml., the usual concentration in routine assays. With amounts of DHAS less than 5 μg. the recoveries were less and not so reproducible.

Isolation of DHAS. To characterize the product formed during the enzymic reaction the isolation of DHAS was attempted. To 10 ml. of buffered ATP and 1 ml. of 2 mM DHA was added 8 ml. of undiluted enzyme solution, and the mixture was incubated for 2 hr. at 37°, after which a further 1 ml. of DHA solution was added. The incubation was continued for a total of 5 hr. and the proteins were then precipitated by the addition of 100 ml. of ethanol. The mixture was filtered, and the filtrate concentrated *in vacuo* to about 2 ml. of a viscous liquid; this was taken up in 10 ml. of methylene-blue reagent and the methylene-blue complex extracted by shaking with three 50 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*, and it gave a small blue residue which was dissolved in 0·5 ml. of ethanol and diluted to 5 ml. with water. To convert the methylene-blue complex into the sodium salt this solution was gently shaken for 15 min. with 200 mg. of Zeo-Karb 225 Na^+ (Permutit Co. Ltd., London), and then filtered. Traces of methylene blue were removed from the filtrate by washing twice with 2 ml. portions of chloroform; traces of chloroform were then removed from the aqueous phase by warming *in vacuo*, and the volume was made up to 10 ml. with water. The resulting solution was shown, as described below, to contain DHAS.

RESULTS

Properties of the enzyme system. Preliminary experiments indicated that the enzyme system forming DHAS had the same general properties as that forming aryl sulphates (Bernstein & McGilvery, 1952*a*, *b*; DeMeio, Wizerkaniuk & Fabiani, 1953). The synthesis of DHAS was ATP-dependent and showed its optimum activity at a concentration of 2 mM ATP (Fig. 1). Higher concentrations of ATP caused a significant inhibition of the reaction, so

that at 8 mM ATP the activity of the enzyme was only some 80% of the maximum. The optimum substrate concentration was approximately 0·04 mM DHA (Fig. 2) and substrate inhibition occurred to a considerable extent. With *epi*androsterone and androsterone the optimum substrate concentration was again 0·04 mM, as shown in Fig. 2. The enzyme system was relatively insensitive to the concentration of SO_4^{2-} ions, as the maximal activity was reached by a concentration of 0·01 M-K_2SO_4, and no decrease in activity was noted at concentrations as high as 0·1 M-K_2SO_4 (Fig. 1). In these experiments, the enzyme preparations used were dialysed against 0·2 M phosphate buffer, pH 6·9, at 0° to reduce the concentration of SO_4^{2-} ions in the enzyme to negligible amounts. Dialysis under these conditions caused about 30% inactivation of the enzyme; dialysis against distilled water caused complete inactivation. The enzyme was thus relatively insensitive to changes in the concentration of SO_4^{2-} ions, and the dialysed enzyme was very unstable. Dialysis was therefore not normally carried out.

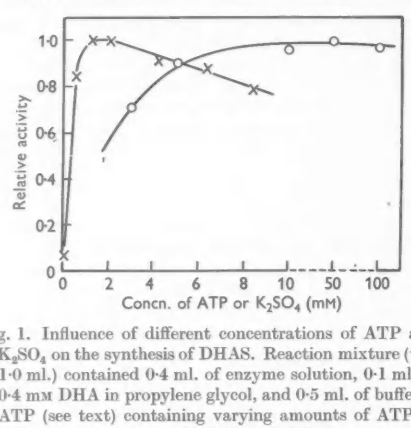

Fig. 1. Influence of different concentrations of ATP and K_2SO_4 on the synthesis of DHAS. Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme solution, 0·1 ml. of 0·4 mM DHA in propylene glycol, and 0·5 ml. of buffered ATP (see text) containing varying amounts of ATP or K_2SO_4. ×, Synthesis of DHAS with different concentrations of ATP, and ○, synthesis of DHAS with different concentrations of K_2SO_4.

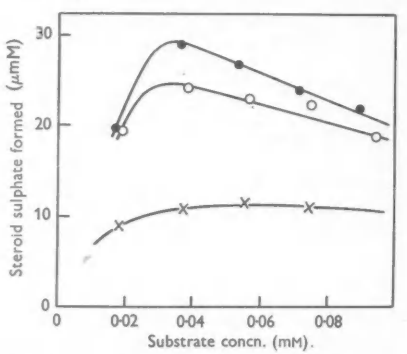

Fig. 2. Effect of substrate concentration on steroid sulphate synthesis. Reaction mixture (vol. 1·0 ml.) contained 0·5 ml. of buffered ATP (see text), 0·4 ml. of enzyme solution and 0·1 ml. of solutions of varying concentration of the steroid in propylene glycol. Incubated for 2 hr. at 37°. ●, DHA; ○, *epi*androsterone; ×, androsterone.

As corticosteroids and salicylates have been reported to inhibit the synthesis of ester sulphates (Kodicek & Loewi, 1955) the effect of these substances on the synthesis of DHAS was investigated. Neither salicylic acid nor acetylsalicylic acid in mM concentrations exerted any effect on the synthesis of DHAS. Cortisone, cortisone acetate, and hydrocortisone all inhibited the formation of DHAS to approximately 20% at mM concentrations, as shown in Table 1.

During the course of the present work it was noted that enzyme preparations from the livers of female rats were consistently more active in the synthesis of DHAS than were those from the livers of male rats. That this is a true difference is readily seen from the following figures. The synthetic activity of preparations from the livers of four male rats ranged from 80 to 165 μmM of DHAS/g. of liver/2 hr., with a mean value of 130 μmM/g., while corresponding preparations from the livers of four female rats had activities ranging from 235 to 325 μmM/g., with a mean of 305 μmM/g.

Specificity of the enzyme system. This was investigated under the general conditions described above, and the results of some of the experiments are shown in Figs. 2 and 3. These figures show the synthesis of the sulphates of a number of androstan-3-ol-17-one isomers. It is obvious that although the enzyme system is relatively non-specific, in that all the isomers are conjugated with

Table 1. *Influence of inhibitors on the synthesis of DHAS*

Reaction mixture (vol. 1·0 ml.) contained 0·4 ml. of enzyme, 0·1 ml. of 0·04 mM DHA, and 0·5 ml. of buffered ATP. The water-soluble inhibitors were dissolved in the buffered ATP and the water-insoluble compounds in the substrate solution. The activity is expressed relative to the controls containing no inhibitor, activity 1·0.

Inhibitor (mM)	Activity
Sodium salicylate (1·0)	0·99
Acetylsalicylic acid (1·0)	0·98
Cortisone (1·0)	0·86
Cortisone acetate (1·0)	0·79
Hydrocortisone (1·0)	0·80
Sodium sulphite (1·0)	1·0
m-Aminophenol (1·0)	0·65
m-Aminophenol (0·5)	0·73

sulphate, there is a considerable variation in the rate of the reaction. The results presented in Fig. 3 show that when the reactions have reached their appropriate steady states the velocities differ in each case. This point is discussed below.

A number of other steroids have been investigated as possible substrates of the reaction, but the results are not quoted in detail as they were obtained under arbitrary conditions, not necessarily optimum for the substrate in question. The substrate concentration was 0·1 mM and the time of incubation 2 hr. Taking the rate of conjugation of DHA under comparable conditions to be 1·00, the rate with pregn-5-en-3β-ol-20-one or 5α-pregnan-3β-ol-20-one as substrate was 1·0, with 5β-pregnan-3β-ol-20-one, 0·6; with oestrone and *epi*testosterone, 0·4; with testosterone, 0·25; with cortisone, 0·1; and with hydrocortisone about 0·05. These results confirm that the sulphate-conjugating system shows a relatively low degree of substrate specificity.

Several compounds other than steroids were also tested as possible substrates under the same conditions, except that the substrate was dissolved in water rather than in propylene glycol. Neither *cyclo*hexanol nor β-phenylethanol nor propylene glycol was conjugated with sulphate at a detectable rate. Salicylic acid reacted very slowly, but phenol was conjugated at a rate comparable to DHA and *p*-nitrophenol at about three times the rate of DHA. When the two latter phenols were dissolved in propylene glycol rather than in water, there was a 40% diminution in the rate of the conjugation of phenol, but with *p*-nitrophenol the rate was unaltered.

Although the properties of the enzyme system forming DHAS suggest that it is identical with the system forming aryl sulphates, direct proof of this assumption has not been possible. Good evidence for the identity of the two systems is given, however, by the fact that *m*-aminophenol, which is known to be rapidly conjugated with sulphate by such preparations (Bernstein & McGilvery, 1952*a*), is a powerful inhibitor of the synthesis of DHAS, a concentration of 0·001 M *m*-aminophenol causing a 40% inhibition of DHAS synthesis (Table 1).

Characterization of DHAS. The product of the enzymic reaction was isolated as described above and was identified as DHAS. Characterization of the compound by classical means was not attempted and the substance was identified by its behaviour on hydrolysis.

Enzymic hydrolysis of the reaction product was carried out with a preparation of the steroid sulphatase of *Patella vulgata* under the conditions already described (Roy, 1954). The reaction product behaved exactly similarly to a standard solution of DHAS when submitted to this enzymic hydrolysis. A further sample was hydrolysed in 0·1 N-H_2SO_4 under the conditions described by Roy (1956), and again the behaviour was identical with that of an authentic sample of DHAS. The results of these experiments are shown in Fig. 4.

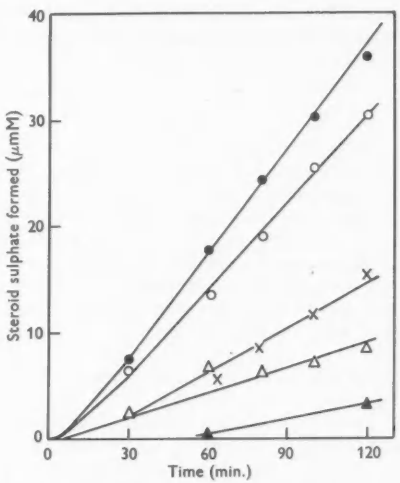

Fig. 3. Course of formation of steroid sulphates. General conditions as in Fig. 2, except that the concentration of the substrate solution was 0·4 mM, giving a final substrate concentration of 0·04 mM, and the time of incubation was varied. △, 5β-Androstan-3β-ol-17-one; ▲, 5β-androstan-3α-ol-17-one. Other symbols as in Fig. 2.

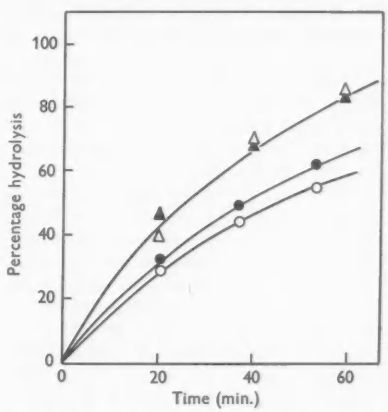

Fig. 4. Hydrolysis of authentic DHAS and DHAS prepared enzymically as described in the text. ▲, Authentic, and △, synthetic DHAS, hydrolysed by 0·1 N-H_2SO_4 at 100°. ●, Authentic, and ○, synthetic DHAS, hydrolysed by steroid sulphatase at pH 4·5 and 37°.

Further proof of the identity of the reaction product with DHAS was obtained by characterization of the steroids produced on acid hydrolysis of the reaction product. A 1 ml. sample of the solution of the product was hydrolysed for 1·5 hr. with 1 ml. of 2N-HCl at 100° and, after cooling, the steroid was extracted with two 5 ml. portions of chloroform. The extract was dried with Na_2SO_4 and taken to dryness *in vacuo*. The residue was dissolved in methanol and submitted to partition chromatography on paper, with the Bush (1952) solvent system *A*.

The steroids produced on the acid hydrolysis of the reaction product were identical with those similarly produced from authentic DHAS, namely, large amounts of chloroandrostenone and small amounts of DHA.

The above experiments were repeated on the reaction product obtained when androsterone was used as substrate. The product behaved then exactly as did authentic androsterone sulphate on hydrolysis by acid, the hydrolysis products being similar in the two cases. The reaction product was not hydrolysed by steroid sulphatase, as was expected from the specificity of that enzyme (Roy, 1956).

There is therefore no doubt that the method described above does indeed measure the rate of formation of steroid sulphates.

DISCUSSION

Although the reaction product has in no case been isolated, these experiments show that when DHA is used as substrate the reaction product is DHAS. The product is an acid-labile conjugate of DHA, which is hydrolysed both by acid and by steroid sulphatase at rates identical with those observed for the corresponding hydrolysis of authentic DHAS (Fig. 4). Further proof that the reaction product is DHAS is afforded by the fact that the reaction is dependent upon the presence of SO_4^{2-} ions in the reaction mixture. The only other product which could conceivably be formed is dehydro*epi*androsterone phosphate, but phosphate esters do not appear to be estimated by the methylene-blue technique (unpublished observations).

The general properties of the enzyme system synthesizing DHAS are similar to those of the system synthesizing *m*-aminophenyl sulphate. Both enzymes are precipitated between 1·7 and 2·3M-$(NH_4)_2SO_4$ and require the presence of 2 mM ATP and 10 mM-SO_4^{2-} ions to exhibit their maximum activity. Again, both enzyme systems are inactivated by dialysis against distilled water and both show typical lag-phase curves for the course of the reaction. It seems reasonably certain that the same enzyme system is involved in the synthesis of both types of compound, but direct evidence for this has not been obtained, except that the synthesis of DHAS is strongly inhibited by *m*-aminophenol. The enzyme system apparently shows a relatively low degree of specificity for steroid substrates, as all sterols so far tested are conjugated with sulphate. It should be pointed out, however, that one enzyme system has not yet been demonstrated to be involved in each case. The only noteworthy result is shown in Fig. 3, which demonstrates that 5β-androstan-3β-ol-17-one is conjugated more rapidly than the corresponding 3α-hydroxy isomer. This is surprising, as the 3β-hydroxyl group is in the axial, therefore hindered, position. In the 5α-androstane series the equatorial hydroxyl group of the 3β isomer reacts considerably more rapidly than the axial group of 3α isomer (Fig. 3), as would be expected.

As shown in Fig. 3, the kinetics of conjugation of the androstanolone isomers were similar to those described by Bernstein & McGilvery (1952*b*) for the conjugation of *m*-aminophenol in that in both there was a lag phase, during which the reaction velocity steadily increased to a maximum value which was maintained for a prolonged period, the linear phase. Bernstein & McGilvery (1952*b*) interpreted these results as indicating the formation of an 'active sulphate' followed by a coupling reaction between this 'active sulphate' and the hydroxyl-containing compound, this second reaction being the rate-limiting one. Segal (1955) developed this theory and provided a mathematical formulation of the results as follows. The two reactions were:

$$A + SO_4^{2-} \xrightarrow{k_1} I,$$

$$I + R.OH \xrightarrow{k_2} R.SO_4^-,$$

the second reaction being rate-limiting. From these assumptions Segal (1955) showed that such a reaction would exhibit a lag phase, the length of which would be governed by k_2, and a linear phase when the system had reached the steady state and when the velocity would be governed solely by k_1. The equations expressing these findings were as follows (Segal, 1955). The velocity at the steady state is

$$\frac{dP}{dt} = k_1, \qquad (1)$$

and the total amount of reaction product (P) formed at any time (t) is

$$P = k_1 t - \frac{k_1}{k_2}(1 - e^{-k_2 t}). \qquad (2)$$

Equation (1) above indicates that the velocity at the steady state is independent of the nature of the substrate, R.OH, as this velocity depends only on k_1. Fig. 3 shows, on the other hand, that the

velocity at the steady state does vary with the substrate. It is therefore obvious that the theory of Segal (1955) is incorrect. The experimental results presented in Fig. 3 can, however, be explained by a very slight modification of the above theory. The modification required is the not improbable one that the first reaction, the formation of 'active sulphate', is reversible. Retaining the nomenclature of Segal (1955) and calling the velocity constant for this reverse reaction k_3, it can be shown that the velocity at the steady state is not independent of the nature of the substrate. As the velocity of the second reaction is rate-limiting, the overall reaction velocity is

$$\frac{dP}{dt} = k_2 I.$$

At the steady state

$$\frac{dI}{dt} = k_1 - k_2 I - k_3 I = 0,$$

$$I = \frac{k_1}{k_2 + k_3},$$

$$\frac{dP}{dt} = \frac{k_1 k_2}{k_2 + k_3}. \qquad (3)$$

This equation (3) shows that the velocity at the steady state depends not only upon k_1 but also upon k_2 and k_3. As k_2 must vary with the substrate used, so also must the velocity at the steady state. This does not alter the rest of the argument of Segal (1955) but only makes the equation describing the total amount of reaction product formed at time t rather more complex, as follows:

$$P = k_1 t - \frac{k_1}{k_2 + k_3}(1 - e^{-(k_2 + k_3)t}). \qquad (4)$$

The form of equation (4) is identical with that of equation (2), but it does not allow the evaluation of k_1 and k_2, as does equation (2), because of the occurrence of a third variable, k_3. None of the constants k_1, k_2 and k_3 is a true velocity constant as they must contain various concentration terms which are not varied under the experimental conditions used. This theory would therefore seem to explain the experimental results more correctly than that of Segal (1955).

There have been many reports in the literature of the inhibition of sulphate-ester synthesis by corticosteroids and by salicylates, but most of these apply to the synthesis of mucopolysaccharides by tissue slices and so are difficult to interpret, as has been stressed by Kodicek & Loewi (1955). The results described above confirm the inhibition of sulphate-ester synthesis by corticosteroids, but show that salicylates have no effect on this process, at least in DHAS synthesis. It must therefore be presumed that previous reports (DeMeio & Tkacz, 1952) of the inhibition by salicylates of aryl sulphate synthesis in liver slices were based on the inhibition of some energy-yielding process involved in the much more complex systems studied by these authors.

One of the most interesting results of the present investigation is the demonstration in rat liver of an enzyme system capable of forming the sulphate esters of a number of steroid alcohols. This shows to be incorrect the statement of Anderton, Smith & Williams (1948) that before a hydroxyl group can be conjugated with sulphate it must have a pK in the range 7–10 and must occur in an enolizing system such as –C:C(OH).C. Of the steroids tested above only oestrone satisfies these conditions, all the other steroids being typical alcohols. It might be argued that the claims of Anderton *et al.* (1948) were based on *in vivo* studies, but there is no reason to suppose that the *in vitro* reactions do not also occur *in vivo*, especially as many of the steroid sulphates have been isolated from urine. It is of interest, however, that none of the simpler alcohols studied showed the ability to conjugate with sulphate, although simple phenols did. It would therefore seem that the claim of Anderton *et al.* (1948) was an oversimplification of the situation, and that the ability of any particular hydroxyl group to conjugate with sulphate is not governed only by its pK value but also by the structure of the molecule as a whole.

SUMMARY

1. A method is described for the assay of steroid sulphate synthesis by soluble enzyme preparations from rat liver. The substrate used is dehydro*epi*androsterone, but the method may be used with any suitable steroid as substrate.

2. The properties of the enzyme system synthesizing dehydro*epi*androsterone sulphate are identical with those of the system which synthesizes aryl sulphates, and it is probable that the same enzyme system is involved in the synthesis of both types of compound.

3. The synthesis of dehydro*epi*androsterone sulphate is inhibited by *m*-aminophenol, cortisone, cortisone acetate and hydrocortisone, but is uninfluenced by salicylates.

4. The substrate specificity of the enzyme system has been investigated and has been shown to be relatively low.

5. An interpretation of the kinetic data is presented.

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The Inhibition of Carbohydrate Metabolism in Ascites-Tumour Cells by Ethyleneimines

By I. M. ROITT

Courtauld Institute of Biochemistry, Middlesex Hospital, W. 1

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The influence of cytotoxic agents upon carbohydrate metabolism has been the subject of several investigations. For example, mustard gas was shown to inhibit glycolysis in Ehrlich rat sarcoma (Jany & Sellei, 1935) and in Jensen sarcoma (Berenblum, Kendal & Orr, 1936). Barron, Bartlett, Miller, Meyer & Seegmiller (1948) found that the respiration of a number of rat tissues was inhibited by methyldi-(β-chloroethyl)amine hydrochloride. Suspensions of Sarcoma 37 obtained from mice after administration of α-peltatin had a decreased ability to utilize glucose anaerobically (Waravdekar, Paradis & Leiter, 1955).

The potent growth-inhibitory properties of the ethyleneimines demonstrated by Hendry, Homer, Rose & Walpole (1951) suggested that a study of the effect of these compounds on carbohydrate metabolism in normal and malignant mammalian cells would be of interest. Cardinali (1954) has already shown that aerobic glycolysis in Rous sarcoma and benzopyrene sarcoma is inhibited by ethyleneimine picrate. In the present work, attention was concentrated mainly on the effect of 2:4:6-triethyleneimino-1:3:5-triazine (TEM) on anaerobic glycolysis in ascites-tumour cells, since this material is convenient for following changes in the concentration of cellular constituents in response to the action of metabolic inhibitors.

A preliminary account of this work has been reported (Roitt, 1955).

EXPERIMENTAL

Ascites-tumour cells. In the majority of experiments, the Krebs II carcinoma in ascites form was used and was maintained in heterozygous albino mice. The animals were killed 7–9 days after inoculation with ascites tumour and the cells were washed with isotonic saline into tubes containing heparin. The ascitic fluid was removed after centrifuging for 5 min. at 2000 *g* and the cells were resuspended in saline, spun at 100 *g* for 3 min. and the bulk of the red cells, still in suspension, removed. The resuspension and low-speed spinning were repeated until contamination with red cells was negligible. The cells were finally suspended in Krebs–Ringer phosphate solution containing 0·1% glucose for aerobic studies, and in Krebs–Ringer bicarbonate containing 0·4% glucose for experiments under anaerobic conditions. The packed-cell volume was determined by centrifuging in a Wintrobe tube for 30 min. at 800 *g*. These preparations contained approximately 5% by number of inflammatory cells, representing less than 1% of the total cell mass.

Respiration and anaerobic glycolysis. The conventional Warburg techniques were used. The media used for studying the metabolism of ascites-tumour cells were also employed for parallel studies on the other mammalian tissues. Substrates were dissolved in isotonic NaCl and the pH was adjusted to 7·4. In a few cases anaerobic lactic acid production was measured (Barker & Summerson, 1941) and shown to be paralleled by the CO_2 evolved. The buffering capacity of TEM was negligible at pH 7·4, so that no correction for retained CO_2 was necessary.

Enzyme preparations

Hexokinase. The activity of a deoxycholate-treated particulate fraction of bullock brain was followed by measuring glucose disappearance (Crane & Sols, 1953; preparation *CD*). Glucose was estimated by the anthrone method (Fairbairn, 1953) after precipitation of phosphates and protein with $Ba(OH)_2$ and $ZnSO_4$.

Triose phosphate dehydrogenase. A dialysed extract of rabbit-muscle acetone powder was used with fructose diphosphate as substrate (Green, Needham & Dewan, 1937). The evolution of CO_2 from a bicarbonate medium

was followed. The enzyme was incubated with the ethyleneimines *in vacuo* to reduce loss of activity by autoxidation.

Phosphofructokinase. An acetone powder of rat brain was extracted with 0·002 M-Na_2HPO_4, pH 7·4. The extract was spun at 18 000 *g* to minimize contamination with particles having high ATPase activity (Muntz & Hurwitz, 1951). The reaction between fructose monophosphate and adenosine triphosphate (ATP) was followed by measurement of acid production manometrically. The enzyme was incubated with fructose monophosphate and the ethyleneimine in Warburg vessels gassed with $N_2 + CO_2$ (95:5); the reaction was started by adding ATP from the side arm. The formation of fructose diphosphate in the reaction was demonstrated enzymically by using the rabbit-muscle preparation of Green *et al.* (1937).

Ascites-cell DPNase. Ascites cells were ground in a Potter-type homogenizer with powdered glass in 0·13% $NaHCO_3$. The resulting suspension was centrifuged at 2000 *g* for 15 min. and the supernatant fluid dialysed against 0·13% $NaHCO_3$ at 0° for 12 hr. The rate of disappearance of added diphosphopyridine nucleotide (DPN) was followed by means of the alcohol dehydrogenase (Racker, 1950) or cyanide (Colowick, Kaplan & Ciotti, 1951) method.

Analytical methods

Ethyleneimine grouping. The reaction of the ethyleneimine ring with $S_2O_3^{2-}$ which results in the equivalent liberation of OH^- may be used as a quantitative method (Ross, 1950). Since many of the solutions to be estimated contained phosphate, a screened indicator consisting of 0·1% aqueous solutions of thymol blue and cresol red (6:1) was used. A solution of 0·4 M-$Na_2S_2O_3$ was boiled to expel dissolved CO_2 and made just alkaline to the screened indicator; 3·0 ml. of this solution was added to the TEM (quantities of the order of 2·0 ml. of 0·01 M), which had also been made alkaline to the indicator, in a small conical flask, and the mixture was placed so that the tip of a Conway microburette dipped below the liquid surface. The mixture was gently heated near to boiling point and 0·2 N acetic acid was run in from the burette to keep the pH slightly on the acid side as the OH^- was generated in the reaction. When the reaction was complete, 0·2 N-NaOH was added until the indicator changed colour. The estimation was accurate to within 3%.

Fructose phosphates. The ascites cells were rapidly spun free of medium and homogenized with cold 10% (w/v) trichloroacetic acid; after centrifuging, the supernatant fluid was neutralized and then frozen and thawed. On spinning down the precipitate a clear supernatant liquid was obtained. The fructose esters were estimated by the method of Roe & Papadopoulos (1954), in which the quantitative separation of the Ba salts is enhanced by the addition of inorganic phosphate. With a synthetic mixture of fructose mono- and di-phosphates, the most consistent results were obtained when the fraction precipitated by Ba from aqueous solution at pH 8·2 (containing the diphosphate probably contaminated with the monophosphate) was redissolved in dilute HCl, excess of Ba acetate added and the pH again adjusted to 8·2. The precipitate was spun down and the supernatant fluids (containing the monophosphate) were combined. This procedure (recommended generally by LePage, 1949) was applied to the cell extracts. The results were confirmed in one case by paper electrophoresis of the extract (see under fructose diphosphate in the Materials section) and estimation of the fructose in successive strips of the paper: the strips were eluted with water, glucose 6-phosphate was oxidized with Br_2 and the fructose determined by heating with the Roe & Papadopoulos reagent.

Other phosphorylated intermediates. The trichloroacetic or perchloric acid cell extracts were neutralized and the Ba salts fractionated as above. P was measured by a micromodification of the Fiske & Subbarow (1925) method. Glucose 6-phosphate was estimated by a modified Hagedorn–Jensen method (Robison & King, 1931), allowance being made for the presence of fructose phosphates. Triose phosphate was estimated as the P liberated in 20 min. at room temp. by N-KOH, and phosphopyruvic acid was estimated as the P liberated by dilute KOH and I_2 relative to the control without I_2. 3-Phosphoglyceric acid (together with 2-phospho- and 2:3-diphospho-glyceric acids) was measured as 'difficultly hydrolysable P'; after 3 hr. hydrolysis with N-HCl at 100° in stoppered tubes, the bulk of the inorganic P was precipitated in 5 min. with magnesia mixture and the organically bound P in the supernatant fluid determined. ATP, adenosine diphosphate and adenylic acid were calculated from the acid-labile P and absorption at 260 mμ. (adenine) of the fractions forming soluble and insoluble Ba salts. Ribose 5-phosphate was estimated as (total ribose of fraction forming soluble Ba salts) – (ribose equivalent of adenylic acid).

Diphosphopyridine nucleotide. The method of Jedeikin & Weinhouse (1955) was followed. The concentration of reduced diphosphopyridine nucleotide was shown to be negligible and therefore only DPN was estimated. The cells (1·0 g.) were rapidly spun down in the cold. 3·0 ml. of boiling 0·04 M-KH_2PO_4, pH 5·4, was added, the cells were rapidly dispersed with a glass rod and immediately added to a Potter-type homogenizer, containing 4·0 ml. of the phosphate buffer and 0·5 g. of powdered glass, which had been heated in a boiling-water bath for at least 4 min. previously. After 60 sec., the contents were homogenized for 30 sec. and cooled in ice; when cold they were homogenized for a further 2 min., made up to a given volume, frozen, thawed and finally spun at 18 000 *g* for 15 min. The DPN content of the clear supernatant fluid was estimated by the method of Racker (1950).

Assay of triose phosphate dehydrogenase in crude cell extracts

The enzyme cannot be assayed in crude extracts by spectrophotometric estimation of the reduction of DPN with triose phosphate, since the glycerol phosphate dehydrogenase present effects the reoxidation of DPNH (Racker, 1947). The basis of the present method is as follows: in the presence of arsenate, triose phosphate formed by the action of aldolase on fructose diphosphate is oxidized by DPN and triose phosphate dehydrogenase to 1-arseno-3-phosphoglyceric acid, which spontaneously undergoes hydrolysis to 3-phosphoglyceric acid. The acid formed liberates CO_2 from the bicarbonate medium. DPNH is reoxidized by pyruvate in the presence of lactic acid dehydrogenase. The triose phosphate oxidation is made rate-limiting by addition of excess of fructose diphosphate, arsenate, DPN, pyruvate, aldolase and lactic acid dehydrogenase.

The ascites-tumour cells (0·5 g.) were dispersed in the incubation medium by homogenizing with powdered glass

for 3 min. The suspension (about 8 ml.) was centrifuged at 18 000 *g* for 35 min. at 0° and the supernatant fluid collected. For the assay, the following were added to each Warburg flask: 1·5 ml. of extract, 0·1 ml. each of solutions of aldolase and lactic acid dehydrogenase (1 part of commercial preparation diluted with 9 parts of water), 0·25 ml. of 0·1 M fructose diphosphate, 0·1 ml. of 1 M sodium pyruvate, 0·1 ml. of 0·3 M-Na_2HAsO_4, pH 7·4, and 0·4 ml. of Krebs–Ringer bicarbonate; the side arms contained 0·3 ml. of 0·17 mM DPN and 0·3 ml. of Krebs–Ringer bicarbonate. The flasks were gassed with $N_2 + CO_2$ (95:5) and allowed to equilibrate at 38°; CO_2 evolution was followed after tipping in. If either DPN or arsenate was omitted from the assay system, CO_2 evolution was negligible, indicating that acid production truly reflected the triose phosphate oxidation. Since relative and not absolute enzymic activities were required, the buffering effects of protein and arsenate could be ignored, as they were the same in both control and TEM-inhibited cells. For absolute determinations it would be necessary to measure lactic acid formation.

The DPNase activity of the cell extracts was low, presumably owing to aggregation of the cytoplasmic particles in a medium containing potassium and their subsequent removal by centrifuging. Where DPNase activity is appreciable, the assay system should include nicotinamide. The rate of acid production was linear over the first 15 min. and, in a typical assay, 100 μl. of CO_2 was evolved during this period. The concentration of TEM was maintained at 0·01 M in the assay system in case dilution affected some reversible TEM-complex.

Materials

Ethyleneimine, 2:4:6-triethyleneimino-1:3:5-triazine, ethyleneiminopropionitrile and *N*-acetylethyleneimine were kindly given by Dr A. L. Walpole and Dr J. A. Hendry (I.C.I. Dyestuffs Division).

Adenosine triphosphate was obtained from Boots Pure Drug Co. as the barium salt, and freed from heavy metals by ion exchange (Polis & Meyerhof, 1947). Calcium fructose diphosphate (L. Light and Co.) was converted into the sodium salt by treatment with sodium oxalate. The solution was analysed by electrophoresis on paper in veronal buffer pH 8·6 (Flynn & de Mayo, 1951), and the spots were localized by the method of Bandurski & Axelrod (1951). The sample was free of monophosphate. Fructose monophosphate was prepared by hydrolysis of the diphosphate (Neuberg, Lustig & Rothenberg, 1943). The product was free of the diphosphate as judged by paper electrophoresis.

*iso*Nicotinamide was prepared from *iso*nicotinic acid via the ethyl ester (Camps, 1902). DPN, aldolase, and lactic and alcohol dehydrogenases were obtained from C. F. Boehringer & Soehne, G.m.b.H. Coramine (*NN*-diethylnicotinamide) was kindly given by Ciba Laboratories, pyridine-3-nitrile by Professor H. McIlwain and nicotinylhydrazide by Dr D. E. Hughes.

The yeast used in these experiments was from The Distillers Co., Ltd.

RESULTS

Effect of TEM on anaerobic glycolysis

A delay in the onset of inhibition of anaerobic glycolysis in Krebs II carcinoma ascites cells by various concentrations of TEM is evident from Fig. 1. This delayed inhibition characterized the effect of TEM on the anaerobic glycolysis of the other tissues studied. The results obtained with Sarcoma 37 cells in the ascites form, slices of rat-brain cortex, liver, hepatoma, kidney cortex and spleen, and yeast cells, are presented in Table 1. With the exception of yeast, brain was the least sensitive to the action of 0·01 M TEM, there being

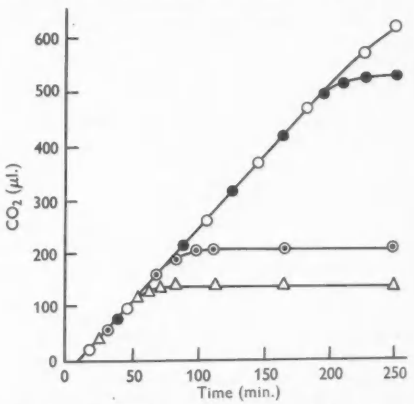

Fig. 1. Effect of TEM on anaerobic glycolysis of Krebs II carcinoma ascites cells. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium. ●, 0·001 M TEM; ⊙, 0·0067 M TEM; △, 0·01 M TEM; ○, control.

Table 1. *Inhibition of anaerobic glycolysis by* 0·01 M *TEM*

Results are the means of duplicates, and are expressed as per cent inhibition compared with control cells without TEM. Slices of rat tissues were used; each Warburg vessel contained between 60 and 100 mg. of fresh tissue suspended in 3 ml. of Krebs–Ringer bicarbonate. In experiments with ascites-tumour cells, 40 mg. of cells was suspended in 2 ml. of Krebs–Ringer bicarbonate, and with yeast, 3 mg. of cells was suspended in a medium containing 0·2% (w/v) $NaHCO_3$ and 0·002 M-NaH_2PO_4. The substrate was 0·4% (w/v) glucose. The gas phase was $N_2 + CO_2$ (95:5). Experiments with mammalian tissues were conducted at 38° and those with yeast at 30°. The reaction was started by tipping 0·2 M TEM (in 0·9% NaCl) from the side arm to give a final concentration of 0·01 M.

Tissue	Time (min.) 0 (%)	60 (%)	90 (%)	120 (%)	270 (%)	300 (%)
S37 Ascites cells	0	50	71	72	96	—
Krebs II ascites cells	0	30	100	—	—	—
Rat-brain cortex	0	0	14	18	62	97
Normal rat liver	0	0	100	—	—	—
Rat hepatoma	0	33	80	82	89	—
Rat-kidney cortex	0	33	52	56	73	81
Rat spleen	0	0	59	72	73	88
Yeast	0	0	0	0	43	43

only very slight inhibition after 2 hr. In contrast, inhibition was virtually complete by 90 min. in Krebs II carcinoma ascites cells (hereafter referred to as ascites-tumour cells), liver and hepatoma.

Ethyleneimine and ethyleneiminopropionitrile resemble TEM in their effects on anaerobic glycolysis. In ascites-tumour cells, anaerobic glycolysis is completely inhibited by 0·023 M ethyleneiminopropionitrile 55 min. after addition of the drug, and by 0·088 M ethyleneiminopropionitrile 120 min. after the addition. *N*-Acetylethyleneimine appears to be less potent.

The anaerobic glycolysis of ascites-tumour cells was studied in mice injected twice daily with 80 μg. of TEM/100 g. of body wt. intraperitoneally, starting 4 days after inoculation with tumour cells. Despite the almost complete suppression of mitosis (one abnormal metaphase was the only mitotic figure seen in 1000 cells) the anaerobic glycolytic rates of cells from animals treated with TEM for 24 and 48 hr. did not differ significantly from the rates of cells from control animals injected with isotonic saline.

Effect of TEM on respiration

Yeast is again insensitive, but in the other cells studied respiration appears to be more susceptible to the action of TEM than is anaerobic glycolysis. The results are summarized in Table 2.

Study of possible factors causing the delay in inhibition

The permeability of ascites-tumour cells to TEM was studied. Within 4 min. of the addition of a comparable volume of cells to medium containing TEM, the concentration of drug in the medium had fallen to a value which accorded with redistribution over the combined volume of cells plus medium. Although rapid penetration of the cells is suggested, adsorption of TEM on the cell surface cannot be excluded. The possibility that TEM reacted with the Krebs–Ringer medium to give a substance with inhibitory properties was studied by incubating TEM with the medium for 5 hr. before addition of the ascites cells. The course of the inhibition was unaltered by this procedure.

It is unlikely that TEM reacts with some cellular constituent to form an inhibitor since addition of intact or homogenized TEM-inhibited ascites cells plus their incubation medium fails to affect the time course of the TEM-inhibition of anaerobic glycolysis in fresh cells.

Table 2. *Inhibition of respiration by* 0·01 M *TEM*

Results are the means of duplicates and are expressed as per cent inhibition compared with control cells without TEM. The experimental details are the same as for Table 1 except for the following: mammalian tissues were suspended in 2 ml. of Krebs–Ringer phosphate; in the experiments with yeast, 30 mg. of cells was suspended in 3·0 ml. of 0·07 M-NaH_2PO_4 buffer, pH 6·8; the substrate was 0·1% (w/v) glucose; the gas phase was O_2.

	Time (min.)			
Tissue	60 (%)	120 (%)	180 (%)	240 (%)
Sarcoma 37 in ascites form	44	86	100	—
Rat-brain cortex	0	16	93	100
Rat-kidney cortex	0	32	68	77
Yeast	0	0	0	—

Effect of TEM on isolated enzyme preparations

In an attempt to elucidate the mechanism of glycolytic inhibition, the action of TEM on certain enzymes concerned in carbohydrate metabolism was studied *in vitro*.

Hexokinase. After incubation for 5 hr. with 0·01 M TEM at 38° the activity of the brain enzyme was slightly higher than that of the control. Similar results were obtained with 0·01 M ethyleneimine.

Triose phosphate dehydrogenase. The activity of a triose phosphate dehydrogenase preparation was inhibited by 20% after incubation for 4 hr. with 0·01 M TEM at 38° and 43% after incubation for 6 hr.

Phosphofructokinase. The effect of incubation with 0·01 M TEM at 38° for different times is shown in Fig. 2. This enzyme is far more sensitive to TEM than either of the other enzymes studied.

Phosphorylated intermediates in TEM-inhibited ascites-tumour cells

Since phosphofructokinase was inhibited *in vitro*, the concentrations of fructose phosphates in TEM-inhibited ascites-tumour cells were estimated to see whether there was an accumulation of the monophosphate as might be expected if phosphofructokinase were blocked. On finding that there was a greater accumulation of fructose diphosphate than of monophosphate in the inhibited cells, the analysis was extended to include other phosphorylated intermediates (Table 3). The ratio of the concentration of fructose monophosphate in TEM-inhibited slices of brain cortex to that in control slices was 2·68; the corresponding ratio for fructose diphosphate was 2·98.

Assay of triose phosphate dehydrogenase in TEM-inhibited ascites-tumour cells

Extracts of cells in which anaerobic glycolysis had been blocked by TEM were able to oxidize triose phosphate at the same rate as the controls without TEM. Further, if the aldolase and lactic acid dehydrogenase additions are omitted from the assay system (see Experimental section), the rates

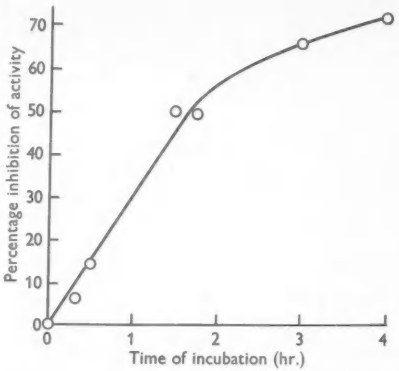

Fig. 2. Effect of incubation with 0·01 M TEM on the activity of a phosphofructokinase preparation from rat brain.

Table 3. *Acid-soluble phosphates in ascites-tumour cells immediately following complete inhibition of anaerobic glycolysis by* 0·01 M *TEM*

Conditions as for Table 1. Results are expressed as the ratio of concentration in inhibited cells to concentration in control cells without TEM.

Phosphate	Ratio
Acid-soluble P	1·0
Inorganic P	1·4
Glucose 6-phosphate	1·3
Fructose 6-phosphate	1·6
Fructose 1:6-diphosphate	3·0
Triose phosphates	1·8
Phosphoglycerate	1·0
Phosphoenolpyruvate	1·0
Adenosine triphosphate	0·17
Adenosine diphosphate	0·39
Adenosine 5′-phosphate	1·5
Ribose 5-phosphate	1·8

of evolution of CO_2 with extracts from TEM-inhibited and control cells are the same. Thus the activities of aldolase, triose phosphate and lactic acid dehydrogenases would appear to be unimpaired by the presence of TEM.

Attempts to reverse the inhibition of anaerobic glycolysis by TEM

The inhibited ascites-tumour cells were washed twice with Krebs–Ringer bicarbonate solution containing 0·4 % glucose, and resuspended in this medium. Under anaerobic conditions there was now a low evolution of CO_2; that this was not due to glycolysis was shown by lactic acid estimations.

Addition of pyruvate, DPN or arsenate either at the beginning of the experiment or when inhibition was complete had no effect. There was a rapid evolution of CO_2 on addition of ATP to the inhibited cells which was unaffected by the presence of 4×10^{-3} M DL-glyceraldehyde (previously shown to inhibit anaerobic glycolysis in the ascites cells by 90 %); the evolution of CO_2 was probably due to the presence of an active ATPase.

DPN concentrations in TEM-inhibited cells

The inability of pyruvate to reverse the inhibition despite the presence of excess triose phosphate and active triose phosphate and lactic acid dehydrogenases suggested that the cells were deficient in DPN. The DPN content of the cells was estimated just before the onset of inhibition, during increasing inhibition and just after the completion of inhibition by 0·01 M TEM in presence of 0·4 % glucose. There was a rapid fall in DPN concentration from 83 % of the control value at 70 min. to 14 % at 90 min. after addition of TEM; inhibition of glycolysis was complete at approximately 80 min.

No DPN could be detected in the medium after inhibition by TEM.

These results may be compared with the rate of fall of DPN content of cells incubated at 38° under anaerobic conditions in the absence of glucose. The negligible evolution of CO_2 indicated that, as expected, glycolysis could not proceed under these conditions. The rate of fall of DPN concentration was considerably lower than that observed in the TEM-inhibited cells; after 80 min., the concentration had fallen only to 59 % of the initial value.

Stability of DPN to the action of TEM

A solution of DPN (0·25 mg./ml.) was incubated at 38° with 0·01 M TEM in 0·01-Na_2HPO_4 buffer, pH 7·2, for 16 hr. The concentration of DPN as measured by the alcohol dehydrogenase method did not change over this period.

Effect of nicotinamide and related substances on the inhibition of anaerobic glycolysis by TEM

The evidence indicating that the inhibition was mediated by a fall in DPN concentration suggested that a study of the effect of nicotinamide on this system might be of value. The results obtained on adding nicotinamide (final concentration 0·01 M) at various times after the addition of TEM are shown in Fig. 3. Nicotinamide protects the system against the action of TEM and, further, it partially restores glycolysis even when added after inhibition is complete. This restoration could be demonstrated by addition of nicotinamide 78 min., but not 95 min., after complete inhibition. The protective effect falls off with time; in the presence of 0·001 M nicotinamide, added at the start of the experiment, the glycolytic rate fell gradually to 10 % of the control value after 3 hr. As shown by titration, TEM did not react with nicotinamide when the two were incubated together at 38°.

A number of substances structurally related to nicotinamide were examined for their protective action against the inhibitory effect of TEM. Of these, none approached the efficiency of nicotinamide in protecting against TEM-inhibition, while only *iso*nicotinamide was able to restore glycolysis in inhibited cells, and that to a minor degree (Table 4). Typical curves are shown in Fig. 4.

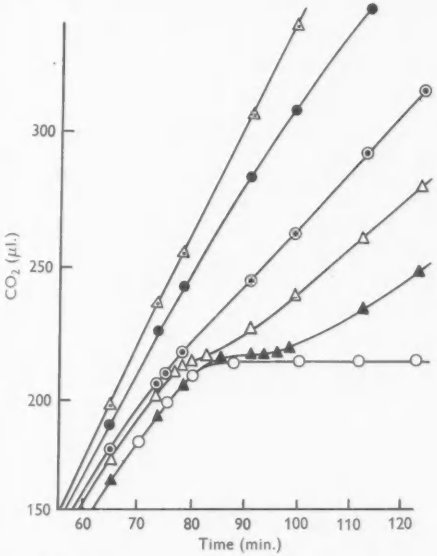

Fig. 3. Effect of 0·01 M nicotinamide on inhibition of anaerobic glycolysis of ascites-tumour cells by 0·01 M TEM. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium; TEM was present in all flasks other than the control. Nicotinamide was added at ●, 0 min.; ⊙, 75 min.; △, 80 min.; ▲, 94 min.; ○, no nicotinamide added; △, control.

TEM and the breakdown of DPN by DPNase

The presence of 0·01 M TEM did not affect the rate of breakdown of DPN by ascites-cell DPNase. When the breakdown was nearly complete, nicotinamide was added (final concentration 0·167 M). Further destruction of DPN was arrested but the concentration of DPN was not increased as might have been expected had a DPN analogue containing TEM been reversibly formed.

DISCUSSION

TEM resembles many of the related cytotoxic agents in its inhibitory action on respiration and anaerobic glycolysis in the tissues studied. Attempts

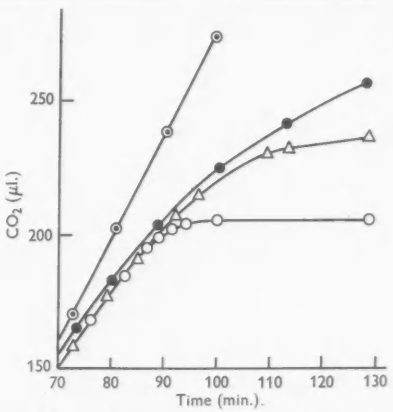

Fig. 4. Effect of *iso*nicotinamide and *iso*nicotinylhydrazide on inhibition of anaerobic glycolysis of ascites-tumour cells by TEM. Each flask contained 0·04 ml. of packed cells suspended in 2 ml. of bicarbonate medium. ●, 0·01 M TEM and 0·01 M *iso*nicotinamide; △, 0·01 M TEM and 0·01 M *iso*nicotinylhydrazide; ○, 0·01 M TEM; ⊙, control, without additions.

Table 4. *Effect of nicotinamide and related substances on the inhibition of anaerobic glycolysis in ascites-tumour cells by* 0·01 M *TEM*

Substance (0·01 M)	Protection against inhibition	Restoration of glycolysis	Reaction with DPN/DPNase*
Nicotinamide	+ + + +	+ + +	+
*iso*Nicotinamide	+ +	+	+
Nicotinylhydrazide	+	−	+
*iso*Nicotinylhydrazide	+	−	+
3-Acetylpyridine	+	−	+
Pyridine-3-nitrile	−	−	−
Nicotinic acid	−	−	−
Nicotinamide methiodide	−	−	−
Coramine	−	−	−
Nicotine	−	−	−
Pyridine	−	−	−

* + signifies ability to exchange with the nicotinamide moiety of DPN in the presence of DPNase. Data taken from Zatman, Kaplan, Colowick & Ciotti (1954).

to localize the site of inhibition by experiments with isolated enzyme preparations revealed that triose phosphate dehydrogenase was relatively stable to the action of TEM. This was unexpected in view of the finding that TEM reacts with the SH-groups of cysteine, glutathione and 2-aminoethanethiol (Roitt, 1954); presumably the SH in the glutathione prosthetic group was partially protected.

In contrast, phosphofructokinase activity was strongly inhibited by incubation with TEM, but analysis of the concentrations of phosphorylated intermediates present in ascites-tumour cells in which anaerobic glycolysis had been completely inhibited by TEM indicated that this enzyme was not blocked. These opposing results cannot be ascribed to the difference in origin of the enzymes since similar changes were observed in TEM-inhibited slices of rat-brain cortex, the tissue from which the isolated preparation was obtained.

The accumulation of both fructose diphosphate and triose phosphate suggests that the reaction affected by TEM is subsequent to the formation of triose phosphate in the breakdown of glucose. The greater accumulation of fructose diphosphate relative to triose phosphate may be connected with the equilibrium in an isolated aldolase system which favours the hexose ester. Similar concentration changes were reported by Holtzer, Haar & Schneider (1955) on inhibition of anaerobic glycolysis in ascites-tumour cells by either iodoacetate or fluoride.

Inhibition by TEM did not alter the concentration of the phosphoglycerate fraction or of phosphoenolpyruvate, suggesting that the reactions by which diphosphoglyceric acid is converted into pyruvate are unaffected. The analytical methods might not detect an accumulation of 1:3-diphosphoglyceric acid occurring at the expense of 2- and 3-phosphoglyceric acids, but the possibility that phosphoglycerokinase was blocked is unlikely since TEM inhibition was not influenced by the presence of arsenate, which permits glycolysis without participation of this enzyme.

Despite the unimpaired activity of triose phosphate and lactic acid dehydrogenases, and the presence of excess triose phosphate in the inhibited cells, the addition of pyruvate failed to restore glycolysis. It was concluded that the concentration of DPN must be inadequate to support glycolysis, and this was confirmed by analysis.

Concurrently with the inhibition of glycolysis the concentration of DPN fell very sharply. There remains the problem of whether the fall in concentration of DPN is the primary cause of glycolytic inhibition or whether some other factor is responsible for blocking glycolysis with consequent lowering in ATP concentration, and hence decreased synthesis of DPN. It was found that when glycolysis was arrested by incubating the cells in a medium devoid of glucose under anaerobic conditions, the rate of fall in concentration of DPN at no time approached that observed in the TEM-inhibited cells, and it must be concluded that the reduction in DPN concentration is probably the primary cause of glycolytic inhibition by TEM. The inhibition of respiration in ascites-tumour cells by TEM accords with this view; the endogenous substrate for the respiration of these cells is probably fat (Slechta, Jakubovic & Sorm, 1955), and DPN provides the common link between anaerobic glycolysis and aerobic catabolism of fat. Instances in which the DPN levels in certain tissues are decreased after the administration of growth inhibitors have been reported. Aminopterin treatment lowered the DPN concentration in rat liver (Strength & Mondy, 1953), while α-peltatin was shown to reduce the DPN concentration in the mouse Sarcoma 37 (Waravdekar *et al.* 1955).

That the inhibitory effect of TEM was mediated via DPNase was suggested by the findings that nicotinamide, 3-acetylpyridine and *iso*nicotinylhydrazide afforded protection against this inhibition in direct relation to their efficiency as DPNase inhibitors. Zatman *et al.* (1954) have shown that many DPNase inhibitors are able to exchange with the nicotinamide moiety of DPN in the presence of DPNase. The DPN analogues so formed usually inhibit the breakdown of DPN; the enzymes from some tissues, although catalysing the exchange, are not themselves inhibited by the analogues. Of the five substances studied which had been shown by Zatman *et al.* (1954) to interact with the DPN–DPNase system, all protected glycolysis against the action of TEM to some degree. Other pyridine derivatives having no effect on the breakdown of DPN by DPNase gave no protection against the action of TEM.

The mechanism by which TEM induces a much greater rate of breakdown of DPN than occurs by the action of DPNase in uninhibited cells remains obscure. There is little or no direct information on the activity of DPNase in intact cells, but it appears to be far less than that observed in disrupted cell preparations. Thus in the above experiments the rate of cleavage of DPN by the ascites-tumour-cell DPNase preparation was at least 10 times the rate observed in intact cells in which DPN synthesis was suppressed by failure of the exergonic reactions. A similar phenomenon was observed in brain slices and dispersions (Gore, Ibbot & McIlwain, 1950). The lower activity of the enzyme in intact cells may be due to (*a*) its localization in a relatively impermeable subcellular structure, (*b*) a low concentration of freely diffusible DPN, or (*c*) the presence of an inhibitor. TEM might act by increasing the accessibility of the enzyme to its substrate, by

increasing the concentration of DPN owing to displacement from combination with apoenzymes, or by reacting with the supposed inhibitor. It is of interest that DPNase activation has been invoked to account for the effect of *Veratrum* alkaloids on cerebral carbohydrate metabolism (Wollenberger, 1955); again, nicotinamide afforded protection against this effect.

Another possibility would be the interaction of TEM and DPN in the presence of DPNase to form a DPN analogue in which TEM replaced nicotinamide. The partial restoration of glycolysis by nicotinamide would then be understandable in terms of resynthesis of DPN by exchange with the TEM bound into the DPN analogue. If such an analogue is formed, the ribose must probably be bound to the nitrogen atom of an ethyleneimine rather than the triazine ring since ethyleneiminopropionitrile inhibits glycolysis in ascites-tumour cells in the same manner as TEM does, and here also the effect is reversed by nicotinamide.

No support for the hypothesis that a DPN analogue was formed could be adduced from experiments in which TEM was incubated with DPN in the presence of ascites-cell DPNase. The breakdown of DPN was unaffected by TEM and further, when the reaction was nearly complete, addition of nicotinamide did not alter the concentration of DPN; the presence of an analogue would have resulted in the resynthesis of DPN by reaction with nicotinamide.

Thus although TEM appears to inhibit glycolysis by increasing the rate at which DPN is broken down, the mechanism by which it does so is not yet known. The role of DPNase in cell metabolism is not fully understood, but the exchange reactions catalysed by the enzyme suggest that it might be implicated in the synthesis of nucleic acids. This would be of considerable interest in view of evidence for the blocking of nucleic acid synthesis by nitrogen mustards and certain other cytotoxic agents (Bodenstein, 1954; Skipper *et al.* 1951).

SUMMARY

1. 2:4:6-Triethyleneimino-1:3:5-triazine (TEM) inhibits respiration and glycolysis in mouse ascites-tumour cells and in a number of rat tissues.

2. Isolated preparations of hexokinase and triose phosphate dehydrogenase are only slightly affected by TEM, but phosphofructokinase is readily inhibited.

3. Fructose diphosphate and triose phosphate accumulate, while the DPN concentration falls sharply, concurrently with the onset of inhibition of anaerobic glycolysis in ascites-tumour cells by TEM.

4. Nicotinamide and a number of DPNase inhibitors protect against this inhibiting effect of TEM.

5. The inhibition is considered to be due to a rapid breakdown of DPN induced by TEM.

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Hydrolysis and Transpeptidation of Lysine Peptides by Trypsin

By Y. LEVIN, A. BERGER and E. KATCHALSKI
Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel

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The study of the tryptic digestion of poly-L-lysine (Katchalski, 1951; Waley & Watson, 1953) has shown that lysine oligopeptides appear during the reaction. A better understanding of the mode of action of trypsin on the high-molecular-weight peptide might be gained by a study of the action of the enzyme on a homologous series of lysine peptides (for a preliminary note see Katchalski, Berger & Levin, 1954). In the present article the behaviour of the following substrates towards trypsin is reported: L-lysine amide, di-L-lysine, tri-L-lysine, di-L-lysine amide, glycyl-L-lysine amide, L-lysylglycine amide, benzoyl-L-lysine, benzoyldi-L-lysine and benzoyltri-L-lysine. In agreement with the results of Waley & Watson (1954), it will be shown that trypsin mediates transpeptidation as well as hydrolysis of many of the substrates investigated. The effect of free α-amino and α-carboxyl groups on hydrolysis and transpeptidation will also be shown.

In the synthesis of the lysine peptides and their derivatives the mixed anhydride method of Wieland & Sehring (1950) as well as the azide method was used. As it has been shown (Vaughan, 1952; Vaughan & Eichler, 1953), that racemization may accompany peptide synthesis when mixed anhydrides of acylated amino acids are used, the azide method was employed in the synthesis of benzoyldi-L-lysine and benzoyltri-L-lysine.

EXPERIMENTAL

Materials

Melting points are uncorrected.

L-Lysine amide dihydrochloride. A stream of dry HCl was passed through a solution of ε-benzyloxycarbonyl-L-lysine amide hydrochloride (Bergmann, Zervas & Ross, 1935) (2 g.) in glacial acetic acid (20 ml.) for 3 hr. at 80°. The precipitation of the L-lysine amide dihydrochloride formed was brought to completion by the addition of anhydrous ether (100 ml.). The precipitate was collected, washed with anhydrous ether and dried *in vacuo* (10^{-2} mm.) at 100° over solid KOH and P_2O_5. Yield, 85%; m.p. 250–260° (decomp.). Waley & Watson (1954) give m.p. 217–218° (decomp.). (Found: C, 33·6; H, 8·1; N, 19·0; amino-N, 12·6; amide-N, 6·8; Cl, 31·6; $C_6H_{17}ON_2Cl_2$ requires C, 33·0; H, 7·9; N, 19·3; amino-N, 12·8; amide-N, 6·4; Cl, 32·5%.)

α-Benzoyl-L-lysine. This compound was prepared according to Ross & Green (1941).

α-Benzoyl-L-lysine amide hydrochloride. This compound was prepared according to Hofmann & Bergmann (1939).

αε-Dibenzyloxycarbonyl-L-lysylglycine methyl ester (I). This compound was prepared from αε-dibenzyloxycarbonyl-L-lysine (4·0 g.) (Bergmann *et al.* 1935), *iso*butyl chlorocarbonate (1·35 g.), triethylamine (2·0 g.) and glycine methyl ester hydrochloride (1·2 g.) in ethyl acetate, following the procedure of Vaughan & Osato (1952). Recrystallization from benzene gave 3 g. of *αε-dibenzyloxycarbonyl-L-lysylglycine methyl ester*, m.p. 122–123°. (Found: C, 62·2; H, 6·8; N, 8·7. $C_{25}H_{31}N_3O_7$ requires C, 61·9; H, 6·4; N, 8·7%.)

αε-Dibenzyloxycarbonyl-L-lysylglycine amide (II). A solution of the ester (I) (0·5 g.) in anhydrous methanol (5 ml.) was saturated with NH_3 at 0° and kept in a tightly closed vessel for 4 hr. at room temp. *αε-Dibenzyloxycarbonyl-L-lysylglycine amide* (0·35 g., m.p. 135°) was obtained after evaporation of solvent and recrystallization from ethyl acetate. (Found: C, 61·3; H, 6·4; N, 12·0. $C_{24}H_{30}N_4O_6$ requires C, 61·3; H, 6·4; N, 11·9%.)

L-Lysylglycine amide dihydrobromide. Compound (II) (10 g.) was treated with anhydrous HBr (30%) in glacial acetic acid (4 ml.) at room temp. (Ben-Ishai & Berger, 1952). The material dissolved with simultaneous evolution of CO_2. The dihydrobromide separated out within a few minutes. After an additional 10 min. the precipitate was washed with glacial acetic acid and finally with ether. Purification was effected by precipitation from a concentrated methanolic solution by means of glacial acetic acid and ether. Yield, 80%. Dried *in vacuo* at 80° over KOH and P_2O_5 before analysis. (Found: N, 15·7; amino-N, 7·45; Br, 43·6. $C_8H_{20}N_4O_2Br_2$ requires N, 15·4; amino-N, 7·7; Br, 43·9%.)

Benzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysine benzyl ester (III). This compound was prepared from benzyloxycarbonylglycine (2·1 g.) (Bergmann & Zervas, 1932), *iso*butyl chlorocarbonate (1·36 g.), triethylamine (2·0 g.) and ε-benzyloxycarbonyl-L-lysine benzyl ester (4·1 g.) (Erlanger & Brand, 1951), in ethyl acetate, following the procedure of Vaughan & Osato (1952). Recrystallization from benzene–ether and from ethyl acetate–light petroleum gave 4 g. of *benzyloxycarbonylglycyl-ε-benzyloxycarbonyl-L-lysine benzyl ester*, m.p. 87–89°. (Found: C, 66·1; H, 6·5; N, 7·7. $C_{31}H_{35}N_3O_7$ requires C, 66·3; H, 6·3; N, 7·5%.)

Benzyloxycarbonylglycyl - ε - benzyloxycarbonyl - L - lysine amide (IV). This compound was prepared from the benzyl ester (III) (2 g.) analogously to the preparation of II. Recrystallized from methanol and ether. Yield 1·5 g., m.p. 134–138° (Bergmann *et al.* 1935 give m.p. 130–134°.)

Glycyl-L-lysine amide dihydrobromide. Compound (IV), on treatment with anhydrous HBr in glacial acetic acid at room temp. analogously to the preparation of L-lysylglycine amide dihydrobromide, was converted into *glycyl-L-lysine*

amide dihydrobromide (yield 78%). It was dried *in vacuo* over KOH and P_2O_5 at room temp. before analysis. (Found: N, 14·8; Br, 44·2. $C_8H_{20}N_4O_2Br_2$ requires N, 15·4; Br, 43·9%.)

Glycyl-L-lysine dihydrochloride. Compound (III) was treated with anhydrous HBr in glacial acetic acid at room temp. for 30 min. according to Ben-Ishai & Berger (1952). The material obtained gave two spots on a paper chromatogram developed with *n*-butanol:acetic acid:pyridine:water (30:6:20:24, by vol.). It was, therefore, treated at room temp. with N-NaOH for 2 hr. to hydrolyse any remaining benzyl ester groups. The hydrolysate was acidified with HCl and evaporated to dryness, and the residue extracted with ethanol. The glycyl-L-lysine dihydrochloride obtained after the evaporation of solvent was found to be chromatographically pure and in the solvent mixture described above showed an R_F value equal to that of a L-lysylglycine prepared according to Bergmann, Zervas, Rinke & Schleich (1934). It also moved as a single spot on paper electrophoresis with a rate equal to that of L-lysylglycine. On acid hydrolysis equimolar amounts of lysine and glycine were obtained.

αε-Dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine benzyl ester (V). Dibenzyloxycarbonyl-L-lysine (1·40 g.) (Bergmann *et al.* 1935) was treated with *iso*butyl chlorocarbonate (1·35 g.), triethylamine (1·00 g.) and ε-benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (4·00 g.) (Erlanger & Brand, 1951) in ethyl acetate (25 ml.) following the procedure of Vaughan & Osato (1952). After the addition of 50 ml. of ethyl acetate, triethylamine hydrochloride was filtered off and extracted with hot ethyl acetate (20 ml.). To the combined ethyl acetate solutions light petroleum (500 ml.) was added and the crystalline precipitate was collected and washed with light petroleum and ether. After recrystallization from ethanol–ether *αε-dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine benzyl ester* (6·8 g. m.p. 139–142°) was obtained. (Found: C, 67·4; H, 6·6; N, 7·2. $C_{42}H_{50}N_4O_9$ requires C, 67·3; H, 6·6; N, 7·3%.)

αε-Dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine amide (VI). This compound was prepared from (V) (2·00 g.) analogously to the preparation of (II). Recrystallization from glacial acetic acid–ether gave 1·70 g. of *αε-dibenzyloxycarbonyl-L-lysyl-ε-benzyloxycarbonyl-L-lysine amide*, m.p. 203–204°. (Found: C, 64·4; H, 6·7; N, 10·5. $C_{36}H_{45}N_5O_8$ requires C, 64·0; H, 6·7; N, 10·4%.)

Di-L-lysine amide trihydrobromide. Compound (VI) was treated with anhydrous HBr in glacial acetic acid at room temp. analogously to the preparation of L-lysylglycine amide dihydrobromide. The product was further purified by precipitation from a concentrated methanolic solution with glacial acetic acid. *Di-L-lysine amide trihydrobromide* was obtained as a white hygroscopic powder in 70% yield. (Found: N, 12·9; amino N, 7·8; amide N, 2·2; Br, 46·3. $C_{12}H_{30}N_5O_2Br_3$ requires N, 13·5; amino N, 8·1; amide N, 2·7; Br, 46·4%.)

α-Benzoyl-ε-benzyloxycarbonyl-L-lysine benzyl ester (VII). ε-Benzyloxycarbonyl-L-lysine benzyl ester hydrochloride (4·0 g.) (Erlanger & Brand, 1951) was treated with benzoyl chloride (2·0 ml.) at 0°, in a mixture of ethyl acetate (20 ml.), 1 N-$KHCO_3$ (20 ml.) and 4 N-NaOH (2·5 ml.). The crystalline product was washed with ether and recrystallized from ethyl acetate and light petroleum. Yield of *α-benzoyl-ε-benzyloxycarbonyl-L-lysine benzyl ester* 2·5 g., m.p. 120°. (Found: C, 70·5; H, 6·1; N, 5·9. $C_{28}H_{30}N_2O_5$ requires C, 70·9; H, 6·4; N, 5·9%.) On treatment with methanol saturated with ammonia (VII) was converted into α-benzoyl-ε-benzyloxycarbonyl-L-lysine amide (Bergmann *et al.* 1935).

α-Benzoyl-ε-benzyloxycarbonyl-L-lysine hydrazide (VIII). Compound (VII) (2·5 g.) was refluxed with hydrazine hydrate (0·5 ml.) in ethanol (10 ml.) for 3 hr. The reaction mixture was cooled and ether (20 ml.) added. The precipitate (1·6 g.) which had a m.p. of 137°, was recrystallized from water. The analytical sample of *α-benzoyl-ε-benzyloxycarbonyl-L-lysine hydrazide* melted at 155°. (Found: C, 63·7; H, 6·4; N, 14·2. $C_{21}H_{26}N_4O_4$ requires C, 63·3; H, 6·6; N, 14·1%.)

α-Benzoyl-di-(ε-benzyloxycarbonyl)-di-L-lysine benzyl ester (IX). A solution of VIII (2·0 g.) in glacial acetic acid (2 ml.) was mixed with ethyl acetate (40 ml.) and 6 N-HCl (10 ml.). To the ice-cooled mixture sodium nitrite (0·40 g. in 10 ml. of water) was added with shaking. The organic layer containing α-benzoyl-ε-benzyloxycarbonyl-L-lysine azide, was separated and the aqueous layer extracted with ethyl acetate (20 ml.). The combined extracts were washed with water, aqueous bicarbonate and water, and finally dried over anhydrous sodium sulphate. The dried azide solution was mixed with an ice-cooled solution of ε-benzyloxycarbonyl-L-lysine benzyl ester (4·0 g.) (Erlanger & Brand, 1951) in ethyl acetate (20 ml.). After standing overnight at room temp., ether (40 ml.) was added and a yield of 2·0 g. of *α-benzoyl-εε-dibenzyloxycarbonyl-di-L-lysine benzyl ester*, m.p. 105°, obtained. Recrystallization from ethanol raised the m.p. to 128°. (Found: C, 68·6; H, 6·4; N, 7·4. $C_{42}H_{48}N_4O_9$ requires C, 68·5; H, 6·6; N, 7·6%.)

α-Benzoyl-di-L-lysine dihydrobromide. Compound (IX) (0·5 g.) was dissolved in glacial acetic acid (10 ml.) and a stream of anhydrous HBr was passed through the solution at 80° during 30 min. Anhydrous ether (20 ml.) was added and the precipitate formed was washed repeatedly with anhydrous ether. It was dissolved in 10 ml. of water, the solution extracted with ether, concentrated to a small volume and *α-benzoyl-di-L-lysine dihydrobromide* precipitated by the addition of absolute ethanol and ether. The white powder thus obtained was washed with ether and dried *in vacuo* over KOH and P_2O_5. (Found: N, 9·8; amino-N, 5·1; Br, 29·8. $C_{19}H_{32}N_4O_4Br_2$ requires N, 10·4; amino-N, 5·2; Br, 29·6%.)

α-Benzoyl-di-L-lysine dihydrochloride. This compound was obtained by catalytic hydrogenation of (IX) in methanol at room temp. and 2 atm. pressure in the presence of Pd (5% on charcoal) and 2 equiv. of HCl. The product obtained had an R_F identical with that of the dihydrobromide.

α-Benzoyl-di-(ε-benzyloxycarbonyl)-di-L-lysine hydrazide (X). This compound was prepared in a 70% yield from (IX) analogously to the preparation of (VIII). After recrystallization from ethanol–ether it had m.p. 185–189°. (Found: C, 63·8; H, 6·7; N, 13·1. $C_{35}H_{44}N_6O_7$ requires C, 63·6; H, 6·7; N, 12·7%.)

α-Benzoyl-tri-(ε-benzyloxycarbonyl)-tri-L-lysine benzyl ester (XI). This compound was prepared in a 58% yield from (X) and ε-benzyloxycarbonyl-L-lysine benzyl ester, analogously to the preparation of (IX). M.p. 120–125°. (Found: C, 67·4; H, 7·0; N, 8·4. $C_{56}H_{66}N_6O_{11}$ requires C, 67·3; H, 6·7; N, 8·4%.)

α-Benzoyl-tri-(ε-benzyloxycarbonyl)-tri-L-lysine (XII). This compound was prepared by alkaline hydrolysis of (XI) in ethanol. It was dissolved in ethyl acetate and

precipitated by light petroleum. M.p. 100–115°. (Found: equiv. wt., 916, by titration with sodium methoxide, with thymol blue as indicator. $C_{49}H_{60}N_6O_{11}$ requires equiv. wt. 909.)

α-Benzoyltri-L-lysine trihydrobromide. Removal of the benzyloxycarbonyl groups of (XII) with anhydrous hydrogen bromide as described in the preparation of (IX) gave *α-benzoyltri-L-lysine trihydrobromide*. Yield 85%. (Found: N, 10·6; amino-N, 5·6; Br, 32·3. $C_{25}H_{45}N_6O_5Br_3$ requires N, 11·1; amino-N, 5·6; Br, 32·0%.)

α-Benzoyldibenzyloxycarbonyl-dilysine benzyl ester was also prepared by coupling ε-benzyloxycarbonyl-L-lysine benzyl ester with the mixed anhydride derived from α-benzoyl-ε-benzyloxycarbonyl-L-lysine and *iso*butyl chloroformate according to the procedure of Vaughan & Osato (1952). The α-benzoyldibenzyloxycarbonyl-di-lysine formed on alkaline hydrolysis was again coupled with *iso*butyl chloroformate and the mixed anhydride obtained reacted with ε-benzyloxycarbonyl-L-lysine benzyl ester. The α-benzoyltri-(benzyloxycarbonyl)trilysine benzyl ester thus obtained was hydrolysed with NaOH and the benzyloxycarbonyl groups of the free acid were removed with anhydrous HBr in glacial acetic acid. The α-benzoyltrilysine isolated from the reaction mixture moved on a chromatogram at a rate identical with that of the α-benzoyltri-L-lysine prepared by the azide method. As it was found, however, that the α-benzoyltrilysine prepared by the azide method is quantitatively digested by trypsin to benzoyllysine and lysyllysine, whereas the α-benzoyltrilysine prepared by the mixed anhydride technique is hydrolysed by the same enzyme only to a small extent, it was concluded that partial racemization occurred during the synthesis of the α-benzoyltrilysine by the mixed-anhydride technique.

Di-, tri-, tetra- and penta-L-lysine. These compounds were prepared according to Waley & Watson (1953).

Enzyme. Crystalline trypsin (50% $MgSO_4$) from Mann Research Laboratories, New York, Inc., was used throughout.

Methods

Enzymic reactions. A weighed amount of substrate (about 5 mg.) was dissolved in 0·25 ml. of 0·1 M phosphate buffer, pH 7·6, and 0·25 ml. of a solution of trypsin (0·004 to 0·8 mg. of N/ml.) in the same buffer was added. The mixture was incubated at 25° or 40°, and samples of 8·0 μl. were withdrawn at given intervals for chromatographic analysis. Blanks were taken on 4·0 μl. samples before the addition of the enzyme.

Qualitative chromatographic analysis. Chromatographic analyses of the enzymic incubation mixtures were carried out according to Waley & Watson (1953), *n*-butanol–acetic acid–pyridine–water (30:6:20:24, by vol.) being used as chromatographic solvent. Table 1 gives the R_F values for the various materials used.

Quantitative chromatographic analysis. Chromatograms were dried in a hood at room temp. for several hours and heated to 100° during 15 min., and the spots were located in ultraviolet light (Fowden, 1951). The paper was sprayed with a 1% solution of KOH in absolute methanol and heated for 15 min. at 60°. The spots, as well as paper blanks, were then cut out and the ninhydrin colour was developed and read (Boissonnas, 1950) on a Hilger Uvispek spectrophotometer H 700/303. Calibration curves giving the optical density at 5700 Å as a function of concentration were obtained from chromatograms containing known amounts of substance whenever the material was found to be chromatographically pure. Otherwise the materials were subjected to large-scale paper chromatography employing about 20 mg. of substance. After elution of the appropriate parts of the paper the concentrations (in mg. of N/ml.) were determined by micro-Kjeldahl analysis, and calibration curves constructed as above.

Paper electrophoresis. Paper electrophoresis analyses were carried out in a Consden & Stanier (1952) apparatus with a potential gradient of 10 v/cm. A 0·1 M acetate buffer (pH 4·0) was used and the electrical current usually passed for 2 hr.

Table 1. *R_F values of reference compounds*

The compounds were chromatographed on Whatman no. 1 paper with *n*-butanol–acetic acid–pyridine–water (30:6:20:24, by vol.).

Substance	R_F	Substance	R_F
L-Lysine	0·20	α-Benzoyl-L-lysine	0·74
Di-L-lysine	0·14	α-Benzoyldi-L-lysine	0·48
Tri-L-lysine	0·10	α-Benzoyltri-L-lysine	0·32
Tetra-L-lysine	0·07	Glycine amide	0·26
Penta-L-lysine	0·05	Glycyl-L-lysine	0·18
L-Lysine amide	0·25	L-Lysylglycine	0·18
Di-L-lysine amide	0·15	Glycyl-L-lysine amide	0·20
		L-Lysylglycine amide	0·20

RESULTS

The following abbreviations are used: Lys for L-lysine and L-lysyl residues, Gly for glycyl residues, and Am for amide groups.

α-Benzoyl-L-lysine. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of benzoyllysine/ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°. Within 48 hr. no reaction products could be detected chromatographically, the spot of benzoyllysine remaining unchanged.

α-Benzoyldi-L-lysine. The reaction mixture contained 2·5 mg. of trypsin, 10 mg. of α-benzoyldilysine dihydrochloride in 1 ml. of 0·1 M phosphate buffer (pH 7·6), and was incubated at 25°. A very slow hydrolytic reaction occurred. Benzoyllysine and lysine could be detected after 24 hr. and their amounts increased on further incubation for 72 hr.

α-Benzoyltri-L-lysine. The course of the reaction is shown in Fig. 1. A significant hydrolysis to α-benzoyllysine and dilysine was detected already within 2 min. After an hour the hydrolysis was almost complete and the only products formed were α-benzoyllysine and dilysine.

Lysine amide. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of lysine amide dihydrochloride/ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°. Although the concentration of substrate was considerably lower than that used by Waley & Watson (1954), a course of reaction similar to that reported by these authors was observed. The main products were lysine and dilysine; traces of trilysine appeared only after several days. The appearance of dilysine and tri-

lysine proves that a transpeptidation reaction occurred. The absence of dilysine amide in most of our experiments shows that this substance, which is probably the first product formed as a result of transpeptidation: $2\ Lys.Am \rightarrow Lys_2.Am + NH_3$, rapidly undergoes further reactions to give either dilysine by hydrolysis or higher lysine peptide amides by transpeptidation. The experiments to be reported below show that $Lys_2.Am$ is rapidly hydrolysed by trypsin. The possible formation of lysine by direct hydrolysis of lysine amide cannot be excluded.

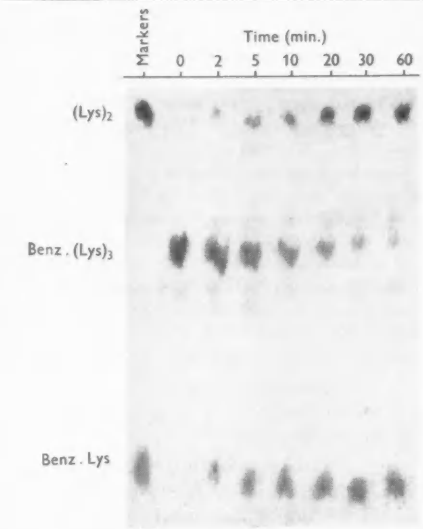

Fig. 1. Chromatogram showing the action of trypsin on α-benzoyltri-L-lysine trihydrobromide. The reaction mixture contained 10 mg. of substrate and 0·25 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

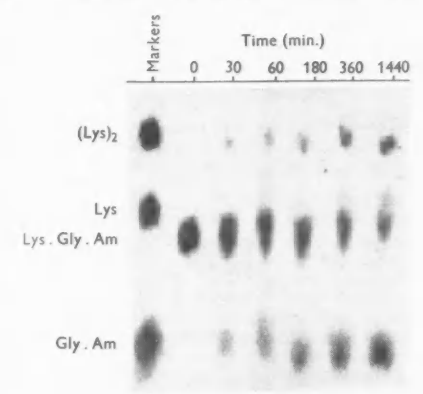

Fig. 2. Chromatogram showing the action of trypsin on L-lysylglycine amide dihydrobromide. The reaction mixture contained 10 mg. of substrate and 2·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

L-Lysylglycine amide. The course of the reaction is shown in Fig. 2. After half an hour of incubation the chromatogram revealed distinct spots of glycine amide and of dilysine, the amount of which increased with time. Lysylglycine amide and lysine could not be separated by paper chromatography. A larger quantity of a substance with an R_F equal to that of lysylglycine amide was therefore separated from a 24 hr. enzymic mixture by paper chromatography; the spot obtained was eluted with 0·1 N acetic acid and analysed by paper electrophoresis. Two distinct spots corresponding to lysine and lysylglycine amide were obtained. The latter moved towards the cathode considerably faster than the former. Additional characterization of the various spots was effected by extraction, acid hydrolysis and subsequent chromatography or paper electrophoresis which allowed the identification of glycine and lysine. Although some intact lysylglycine amide remained in the reaction mixture even after an incubation period of 48 hr., the amounts of glycine amide, lysine and dilysine increased and traces of trilysine appeared.

The formation of dilysine during the reaction suggests a transpeptidation reaction followed by a rapid hydrolysis:

$$2\ Lys.Gly.Am \rightarrow Lys_2.Gly.Am + Gly.Am,$$
$$Lys_2.Gly.Am \rightarrow Lys_2 + Gly.Am.$$

$Lys_2.Am$ (see below), as well as $Lys_2.Tyr.Am$ (cf. Waley & Watson, 1954), is rapidly hydrolysed by trypsin to Lys_2 and NH_3 or Lys_2 and Tyr.Am respectively. This may explain the absence of $Lys_2.Gly.Am$ on the chromatogram. Lysine may be formed either by direct hydrolysis of Lys.Gly.Am, or from Lys_2 as discussed above.

Glycyl-L-lysine amide. The behaviour of glycyl-L-lysine amide towards trypsin is given in Fig. 3. Rapid hydrolysis of the substrate occurred, yielding glycyllysine exclusively. Within 24 hr. the hydrolysis was almost complete. The spot of glycyllysine formed yielded after elution and total acid hydrolysis equimolar amounts of glycine and lysine which were identified by paper chromatography, with butanol–acetic acid–water (4:1:5, by vol.) as developing solvent, or by paper electrophoresis.

Di-L-lysine. The reaction mixture contained 2·5 mg. of trypsin and 10 mg. of dilysine trihydrochloride/ml. of 0·1 M phosphate buffer (pH 7·6) and

was incubated at 25°. The results obtained were in agreement with those given by Waley & Watson (1954), i.e. similar amounts of lysine and trilysine were formed.

Di-L-lysine amide. The action of trypsin on dilysine amide is given in Fig. 4. The chromatogram revealed that dilysine amide gives on incubation with trypsin mainly dilysine; however, lysine amide, lysine, as well as trilysine and probably traces of tetralysine, appeared in the mixture. It was observed that tetralysine disappeared after 24 hr. and the amount of lysine amide formed was considerably larger than that of lysine.

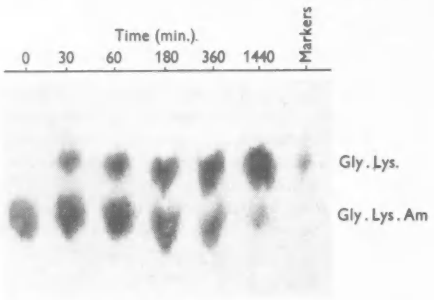

Fig. 3. Chromatogram showing behaviour of glycyl-L-lysine amide dihydrobromide towards trypsin. The reaction mixture contained 10 mg. of substrate and 0·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

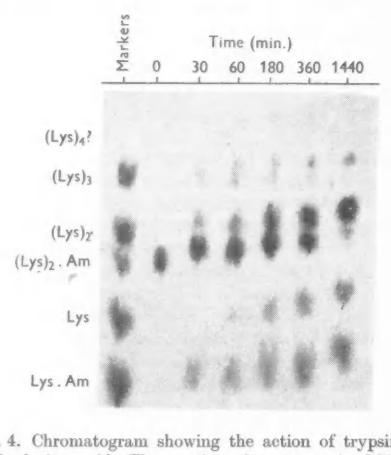

Fig. 4. Chromatogram showing the action of trypsin on di-L-lysine amide. The reaction mixture contained 10 mg. of dilysine amide trihydrobromide and 0·5 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

A quantitative analysis of the products present in an incubation mixture after 18 hr. is given in Table 2. The amounts of dilysine amide, dilysine, lysine amide, lysine and trilysine were determined by quantitative chromatography. For the determination of the amount of ammonia liberated 0·4 ml. of the initial reaction mixture, containing 2·38 mg. of dilysine amide trihydrobromide, was transferred into the outer compartment of a Conway dish which contained 0·1 N-HCl in the centre compartment; the dish was closed and incubated for 18 hr. at 25°. At the end of this period 1 ml. of saturated K_2CO_3 was added to the outer compartment, the dish closed and left for 3 hr. at room temperature. The excess of HCl was titrated with 0·1 N-NaOH, with screened methyl red as indicator. A duplicate analysis of each quantitative determination was carried out, and the averages thus obtained are reported in Table 2.

As dilysine amide gives mainly dilysine and ammonia, it seems that the hydrolytic reaction

$$Lys_2.Am \rightarrow Lys_2 + NH_3$$

plays a major part in the enzymic reaction under consideration. The direct hydrolysis

$$Lys_2.Am \rightarrow Lys + Lys.Am$$

seems less probable as the amounts of lysine and lysine amide formed are small, and the molar concentration of lysine amide exceeds considerably that of lysine. The formation of trilysine and tetralysine indicates that a transpeptidation reaction also occurs. The appearance of trilysine may be explained, for example, by the following two successive steps:

$$2Lys_2.Am \rightarrow Lys_3.Am + Lys.Am,$$

and

$$Lys_3.Am \rightarrow Lys_3 + NH_3.$$

Trilysine amide may, of course, be hydrolysed by other routes such as

$$Lys_3.Am \rightarrow Lys_2 + Lys.Am.$$

These reactions may also explain the relatively high concentrations of lysine amide as compared to those of lysine.

Table 2. *Quantitative analysis of the reaction products formed on incubation of dilysine amide with trypsin*

[*] Incubation mixture composed of 5·95 mg. (11·5 μmoles) of dilysine amide trihydrobromide and 0·5 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6). Incubation period 18 hr. at 25°.

Material	Concentration (μmoles/ml.)
Dilysine amide	2·0
Dilysine	6·5
Lysine amide	1·9
Trilysine	0·5
Lysine	1·1
Ammonia	7·2

The appearance of tetralysine may be explained by a transpeptidation reaction:

$$2Lys_2 . Am \rightarrow Lys_4 . Am + NH_3,$$

followed by a hydrolytic reaction:

$$Lys_4 . Am \rightarrow Lys_4 + NH_3.$$

As tetralysine disappears rapidly in the presence of trypsin, its concentration in the reaction mixture is small.

Tri-L-lysine. Fig. 5 shows that tetra-, penta-, and hexa-lysine appear in an incubation mixture of trilysine and trypsin within a few minutes, and their concentration stays practically constant for several hours. The concentration of trilysine decreases, while that of dilysine increases, during this period. A strikingly small amount of lysine was formed.

A quantitative chromatographic analysis of an incubation mixture of trilysine and trypsin at different time intervals is given in Table 3. The amounts of tetra-, penta- and hexa-lysine were too small for a reliable quantitative analysis.

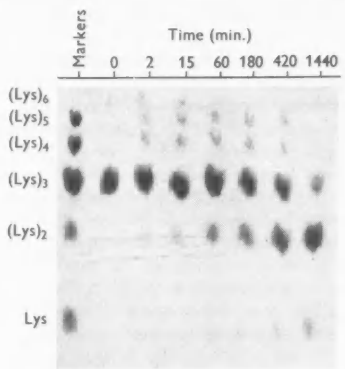

Fig. 5. Chromatogram showing the action of trypsin on tri-L-lysine tetrahydrochloride. The reaction mixture contained 10 mg. of substrate and 0·5 mg. of enzyme in 1 ml. of 0·1 M phosphate buffer (pH 7·6) and was incubated at 25°.

The appearance of tetra-, penta- and hexa-lysine in the reaction mixture proves that a transpeptidation reaction took place. Their formation may be explained by reactions such as:

$$2Lys_3 \rightarrow Lys_4 + Lys_2, \quad Lys_4 + Lys_3 \rightarrow Lys_5 + Lys_2$$
$$2Lys_3 \rightarrow Lys_5 + Lys, \quad Lys_4 + Lys_3 \rightarrow Lys_6 + Lys$$
$$Lys_5 + Lys_3 \rightarrow Lys_6 + Lys_2.$$

In all our experiments (e.g. Table 3) the relative amounts of lysine were small and the trilysine which disappeared from the reaction mixture was transformed almost quantitatively into dilysine. This indicates that a direct hydrolysis of trilysine to dilysine and lysine does not proceed to a considerable extent. As the higher lysine peptides are hydrolysed more rapidly than the lower ones (Waley & Watson, 1953) it seems probable that the tetralysine and the higher lysine peptides serve as intermediates in the transformation of trilysine to the terminal products of the enzymic reaction.

Transpeptidations of trilysine leading to the liberation of dilysine appear to dominate those leading to the formation of lysine. The tetralysine which was formed by transpeptidation may give dilysine by hydrolysis: $Lys_4 \rightarrow 2Lys_2$. The pentalysine, on the other hand, may give dilysine and trilysine. A more complicated route of hydrolysis would be expected for hexalysine.

As the concentration of trypsin in these experiments was considerably lower than that required to effect noticeable cleavage of dilysine, it seems that the amount of dilysine accumulated approximates the amount formed. This conclusion is supported by the small amounts of lysine found in the tryptic digests of trilysine.

The results show that the cleavage of trilysine by trypsin involves a transpeptidation reaction leading to the formation of higher lysine peptides, which yield mainly dilysine on hydrolysis.

DISCUSSION

The minimum structural requirements for trypsin substrates were summarized by Neurath & Schwert (1950) and by Green & Neurath (1954). It was shown that the only peptide or ester bonds

Table 3. *Quantitative analysis of an incubation mixture of trilysine and trypsin*

The incubation mixture contained 10·23 mg. (18·7 μmoles) of trilysine tetrahydrochloride and 0·26 mg. of trypsin in 1 ml. of 0·1 M phosphate buffer (pH 7·6), and was incubated at 40°. Samples (8·2 μl.) were withdrawn at given intervals and the amounts of trilysine, dilysine and lysine determined as described above. Concentrations are given in μmoles of lysine residue × 10^2 per 8·2 μl. of reaction mixture.

	Time (min.)						
Material	0	15	45	100	180	360	1440
Trilysine	46·2	44·0	40·0	37·8	35·8	29·7	18·4
Dilysine	0·0	1·6	3·9	8·2	10·0	17·1	28·8
Lysine	0·0	0·0	0·0	0·5	1·0	1·6	2·2
Trilysine decomposed	—	2·2	4·0	8·4	10·5	16·5	27·9

which are hydrolysed are those in which the carbonyl group is contributed by arginine or lysine, possessing a free ϵ-amino or a free δ-guanido group respectively. The blocking of the α-amino group of lysine or arginine by an acyl group facilitates considerably hydrolysis at alkaline pH values. Our findings with benzoyltrilysine, benzoyldilysine and benzoyllysine clearly indicate that in these substrates, in which no transpeptidation takes place, an α-carboxyl group adjacent to a lysyl peptide bond inhibits its tryptic digestion. Benzoyltrilysine which contains two lysyl peptide bonds, the first adjacent to a free α-carboxyl group and the second remote from it, is hydrolysed rapidly, in the presence of trypsin, at the remote peptide bond to give benzoyllysine and dilysine. Benzoyldilysine, on the other hand, which contains only one lysine bond adjacent to a free α-carboxyl group, is practically resistant to tryptic digestion.

The elucidation of the effect of an α-amino group adjacent to a lysyl peptide bond on the availability of this bond to tryptic digestion is considerably more difficult than in the case of the carboxyl group, since the presence of a free amino group enables transpeptidation to occur, leading to the formation of new compounds, some of which might be formed by direct hydrolysis of the substrate. The simplest compound for the investigation of the influence of an adjacent α-amino group on the rate of hydrolysis of a lysyl peptide bond is lysine amide. The evidence advanced by Waley & Watson (1954), as well as the results reported above, show that the disappearance of lysine amide, in the presence of trypsin, takes place to a significant extent only at relatively high enzyme concentration. Furthermore, it seems probable that, under the experimental conditions used, most of the lysine formed was obtained as a result of transpeptidation. These observations support the conclusion that a free α-amino group adjacent to a lysyl amide bond retards its hydrolysis by trypsin. The findings of Waley & Watson (1954) that dilysine ethyl ester is rapidly hydrolysed in the presence of low concentrations of trypsin to dilysine and ethanol, as well as our findings that dilysine amide is hydrolysed by trypsin mainly to dilysine and ammonia, also indicate that while the amide or ester bonds which are remote from the free α-amino group of the dipeptide undergo relatively rapid hydrolysis, the lysyl amide bonds which are adjacent to the α-amino group remain intact or undergo only slow hydrolysis. The observation of Waley & Watson (1954) that dilysyltyrosine amide yields in the presence of trypsin mainly dilysine and tyrosine amide, as well as our own observation that glycyllysine amide is hydrolysed considerably faster than lysylglycine amide, the former yielding glycyllysine and ammonia and the latter lysine and glycine amide, are also in accord with the interpretation given above for the inhibitory effect of the α-amino group. The findings of Werbin & Palm (1951) and Goldenberg & Goldenberg (1950) that lysine and arginine esters are hydrolysed rapidly by trypsin seem to contradict the conclusions drawn concerning the inhibitory action of the α-amino group. However, esters usually undergo tryptic hydrolysis considerably more rapidly than the respective amides and the optimum tryptic hydrolysis of the arginine methyl ester takes place at pH 5·8, whereas all the enzymic experiments reported in the present paper were carried out at pH 7·6. The data of Goldenberg & Goldenberg (1950) show that at this alkaline pH the tryptic hydrolysis of arginine ester is very slow.

The only peptide bond in lysyllysine is adjacent to a terminal α-carboxyl as well as a terminal α-amino group; as expected, it is hydrolysed extremely slowly by trypsin. At relatively high enzyme concentrations the dipeptide gives lysine and trilysine as a result of transpeptidation. In trilysine two peptide bonds are present, one adjacent to a free terminal carboxyl group and the other to a free terminal α-amino group. The tryptic hydrolysis of both peptide links should therefore be inhibited. The observation that trilysine is transformed almost quantitatively into dilysine, as well as the observation that higher lysine peptides appear in the enzymic reaction mixture, show that the cleavage of trilysine proceeds mainly through an intermediate transpeptidation reaction and not by direct hydrolysis. With tetralysine only one peptide bond is remote from an α-amino group and an α-carboxyl group. It would therefore be expected that the tetrapeptide would be hydrolysed by trypsin mainly to dilysine. This conclusion was confirmed experimentally. The appearance of lysine and trilysine in small quantities in the tryptic digest of this peptide may be explained either by transpeptidation or by direct hydrolysis. Pentalysine possesses two peptide bonds which are not affected by the two terminal groups, and their hydrolytic cleavage should obviously lead to the formation of equimolar amounts of dilysine and trilysine. In fact Waley & Watson (1953) found that at low enzyme concentration, when the transpeptidation reactions of dilysine and trilysine in the presence of trypsin are negligible, pentalysine yielded the di- and tri-peptide in equimolar amounts.

In order roughly to estimate the relative rates at which the various substrates are degraded by trypsin, the time $t_{\frac{1}{2}}$, required for the disappearance of approximately 50% of substrate was determined chromatographically in a number of cases. The molar concentration of the lysine residue in each of the substrate solutions tested was 50 μM.

The results given in Table 4 confirm the general conclusions drawn above. In addition, it is demonstrated that benzoyltrilysine is hydrolysed at a rate comparable with that of tetralysine, and considerably more rapidly than benzoyllysine amide. This suggests that the number of amide bonds in the substrate has a pronounced influence on the rate of tryptic digestion.

In the present work, as well as in the work of Waley & Watson (1954), transpeptidation was recognized by the formation of products which cannot be derived from the substrate by direct hydrolysis. The appearance of peptides with a number of lysine residues greater than that present in the original substrate, or in some cases the appearance of peptides with a sequence of amino acids different from that to be found in the substrate, proved that transpeptidation occurred. All the substrates in which transpeptidation reactions occurred contained a free α-amino group. The blocking of the α-amino group by a benzoyl group eliminated transpeptidation. This suggests that the transpeptidation reactions investigated are of the carboxyl-transfer type, in which the carboxyl moiety of the donor peptide is transferred to new linkage with the free amino group of the acceptor (Hanes, Hird & Isherwood, 1950). In several transpeptidation reactions with lysine peptides or their derivatives the substrates served simultaneously as donors and acceptors. Transpeptidation occurs at a relatively low concentration of peptides participating in the reaction. Hydrolysis, on the other hand, takes place between a substrate at a relatively low concentration, and water at high concentration (55·5 moles/l.). The appearance of transpeptidation products in the reaction mixture indicates therefore that the specific rate constants of the transpeptidation reactions are considerably higher than the specific rate constants of hydrolysis.

Table 4. *Time of disappearance of 50 % substrate by tryptic digestion*

The reaction mixtures tested were composed of the substrate in a concentration of 50 μM lysine residue, and trypsin, in a concentration specified below, in 0·1 M phosphate buffer (pH 7·6). Incubation temp. 25°.

Substrate	Enzyme concn. (mg. of protein/ml.)	$t_{\frac{1}{2}}$ (min.)
α-Benzoyltrilysine	0·025	40
α-Benzoyllysine amide	0·25	120
Dilysine amide	0·25	288
Glycyllysine amide	0·25	258
Lysylglycine amide	5·00	230
Lysine amide	5·00	540
Tetralysine	0·025	100
Trilysine	0·26	720

SUMMARY

1. The synthesis of L-lysine amide, L-lysylglycine amide, glycyl-L-lysine amide, di-L-lysine amide, α-benzoyldi-L-lysine and α-benzoyltri-L-lysine is described.

2. α-Benzoyltri-L-lysine is rapidly hydrolysed by trypsin to α-benzoyllysine and dilysine. α-Benzoyldi-L-lysine is almost resistant to trypsin.

3. Chromatographic analysis showed that although glycyl-L-lysine amide is hydrolysed rapidly by trypsin to glycyllysine and ammonia, L-lysylglycine amide is attacked by trypsin slowly, yielding dilysine in addition to glycyllysine amide and lysine.

4. The chromatographic analysis of the incubation mixtures with trypsin of L-lysine amide, di-L-lysine amide and tri-L-lysine proved that transpeptidation as well as hydrolysis take place.

5. A quantitative chromatographic analysis of the reaction products formed on incubation of tri-L-lysine with trypsin showed that the tripeptide is transformed almost quantitatively into dilysine. Tetra-, penta-, and hexa-lysine appear in the incubation mixture.

6. The inhibitory effect of α-amino and α-carboxyl groups on the trypsin-catalysed hydrolysis of an adjacent lysyl peptide bond is discussed.

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Some Components of Tobacco Mosaic Virus Preparations made in Different Ways

By N. W. PIRIE
Rothamsted Experimental Station, Harpenden, Herts.

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Tobacco mosaic virus (TMV) is being studied in many different laboratories for many different reasons, and with results that are not always identical. Some of the diversity is probably caused by the existence of many virus strains, but much of it comes from differences in the conditions under which the host plants are grown and in the technique used for isolating the virus. Bawden & Pirie (1945) demonstrated that several different types of fraction could be made from infected leaves, each containing material related serologically to TMV but differing in infectivity, physical properties and chemical composition.

The different fractions in extracts of the infected leaf differ in their rate of sedimentation in the ultracentrifuge and in the readiness with which they form a coherent pellet in the tube. If therefore the leaf extract is ultracentrifuged for the minimum time needed to sediment most of the virus, and if as much as possible of the supernatant fluid is poured off, the preparation will contain only part of the total material related to TMV, but that part will be relatively uniform because it will contain only one of the fractions described by Bawden & Pirie. The preparations of TMV used in most laboratories are of this type, but even with them there are some inconsistencies, and changes go on during storage.

The object of this paper is to describe some of these changes and to suggest techniques for testing TMV preparations to determine the extent to which they have occurred. These changes had gone nearly to completion in preparations made by the original brutal methods (Bawden & Pirie, 1937*a*, 1943); the gentler ultracentrifugal methods that are now generally used leave components in the preparations that are not essential for infectivity. Significance can be attached to differences in the physical properties or analytical composition of virus strains only if it is certain that all are in the same state. It is clearly convenient that this state should be the simplest with all dispensable material removed, even though there is reason to think (cf. Pirie, 1949, 1953) that complexes between the simplest form of TMV and other materials are not artifacts but exist in the infected plant.

EXPERIMENTAL

Materials and methods

All the preparations of TMV used in these experiments were the pellets made when obtaining ultracentrifuge supernatant fluid for an examination of the properties of the very slowly sedimenting forms of TMV. The technique of growing and harvesting the plants is described in another paper (Bawden & Pirie, 1956). Three virus strains—type strain, *Datura* and U2—were used; their relationships to one another are discussed in that paper. The technique of mincing and ultracentrifuging was that used for making normal leaf nucleoprotein (NP) (Pirie, 1950). Unless otherwise stated, the preparations were sedimented for 30 min. at 65 000–80 000 *g*.

The serological activities and infectivities of these preparations were measured (Bawden & Pirie, 1956) and no preparation used here was less than half as active as the best preparations. Tests were also made on material that had been exposed to the various forms of incubation. These showed that there had been no significant fall in infectivity, and the serological activities, when measured after the aggregation procedure described by Bawden & Pirie (1956), were also unaltered.

The techniques of precipitating nucleic acid with uranyl nitrate and trichloroacetic acid (TCA) and of determining total P were those described by Holden & Pirie (1955*c*).

The measurements of ultraviolet absorption were made at about 20° in 1 cm. quartz cells on a spectrophotometer (Unicam Instrument Co.).

Yeast nucleic acid was used as substrate in the determinations of nuclease activity, and was made by the method of Holden & Pirie (1955*b*).

An air-driven ultracentrifuge of the type described by Masket (1941) was used; in this the tubes are inclined at only 10° to the axis. Sedimentation is therefore effectively complete when the material has moved about 1 cm. across the tube rather than the much greater distance down it. The rotor was cooled to 0° immediately before each run, and was still at less than 5° at the end of it.

RESULTS

Determination of the nucleic acid content

General aspects of nucleoprotein fission. If preliminary treatments, followed by adequate tests, show that a TMV preparation is substantially free from phospholipid, normal leaf nucleoprotein, deoxyribonucleic acid, etc., clearly its P content can be used as a measure of the amount of ribonucleic acid present. In practice the absence of presumed contaminants cannot be demonstrated satisfactorily, and it is necessary to separate the nucleic acid or its breakdown products and to attempt their identification. This is one facet of the general problem of determining the nucleic acid content of tissues, but with TMV special factors arise. Nucleic acid is more easily separated from TMV than from many other ribonucleoproteins (Pirie, 1954; Holden & Pirie, 1955*b*). This makes it possible to differentiate between ribonucleic acids occurring in the same preparation but linked in different ways. It also makes it easier to separate the nucleic acid in a macromolecular form, and when in this form it can be further characterized by following the effect of enzyme preparations on it. This possibility is lost if the nucleic acid has been extensively degraded by separation from the protein with alkali, TCA or $HClO_4$, as in the methods of Schmidt & Thannhauser (1945), Schneider (1945) and Ogur & Rosen (1950). These methods are obviously convenient in use and are well adapted to routine estimation, but the advantages are gained at the expense of some loss in discrimination.

Some possible methods for splitting TMV have already been discussed (Pirie, 1954). Holden & Pirie (1955*b*) found that, after fission by boiling, only 2–3 % of the total P remained associated with the main protein coagulum. Not all the virus protein is precipitated with the coagulum, and treatments with other deproteinizing agents, e.g. 'colloidal iron' (Bawden & Pirie, 1937*a*) or chloroform and pentanol (Holden & Pirie, 1955*b*), are needed to remove it completely. This non-uniform behaviour of the protein suggests that even the most highly purified preparations of TMV may contain more than one type of protein, and this should be borne in mind in investigations of the structure or amino acid composition of TMV. It is also of interest in conjunction with the suggestion made by Elson & Chargaff (1955) that an intact ribonucleic acid chain is normally associated with a polypeptide chain, and that the double system is the fundamental structural particle. The amount of protein that remains soluble after heating TMV under suitable conditions is 5–8 % of the total, so that protein and nucleic acid are present in the solution in approximately equal amounts, which is in accord with Elson & Chargaff's suggestion.

Nucleic acids have their maximum absorption at about 260 mμ., whereas most proteins have it at 280 mμ. The ratio of density at 260 mμ. to density at 280 mμ. can therefore be used as a measure of the amount of protein present. Thus for purified nucleic acid the ratio is nearly 2, whereas it is 1·65–1·8 on these heated fluids. This is also the range found with extracts made by heating with $HClO_4$, so that, although there is some evidence of contamination, it is not serious and is no worse after heating than after $HClO_4$ extraction.

Fission by heating

The conditions of heating are not critical. The solution must not be acid, or nucleic acid is lost on the protein, and some salt must be present, or the protein will not coagulate (Bawden & Pirie, 1937*a*). A convenient mixture contains 2·5 g. of virus/l., 0·005 M citrate buffer (pH 6) and 0·025 M-NaCl. This is heated in a boiling-water bath for 3 min., cooled and centrifuged, and the precipitate is stirred up with half the volume of 0·025 M-NaCl and centrifuged again. The fluids are mixed for P determination and measurement of ultraviolet absorption; total P is determined on the washed precipitate. It is necessary to work with about 10 mg. of TMV, i.e. 4 ml. of solution, if the figure for the P content of the precipitate is to have any significance. If significance is being attached only to the density at 260 mμ. and to the P content of the fluid, one-fifth or one-eighth of this weight of virus is sufficient. Preparations of the different strains of TMV that have been used behave differently on coagulation. Thus the *Datura* strain has been found to flocculate more quickly than the type strain, and strain U 2 coagulates more slowly and remains gelatinous and bulky. It cannot therefore be washed satisfactorily, so that it carries down more P than the proteins from the other strains.

In spite of these differences in the texture of the denatured protein, nucleic acid appears to separate similarly from all the strains examined, provided that they have received the same type of pretreatment. But the nucleic acid does not separate completely when preparations contain significant amounts of the labile P that is discussed below.

Incubation with citrate removes this labile P, and after this treatment the coagulum is free from P. If, as is likely, the labile P comes from NP associated with the TMV, this behaviour is to be expected because, even under the best conditions that could be found (Holden & Pirie, 1955*b*), protein and nucleic acid did not separate completely when NP was boiled. These phenomena are probably responsible for much, if not all, of the effect described by Cooper (1953) and Cooper & Loring (1954). They found that only two-thirds of the nucleic acid separated from the protein when their preparation of TMV was denatured by heat in the fresh state, but that separation was more complete when the preparation had aged for a few weeks. In the absence of any other evidence it seems probable that the fresh preparations contained a considerable amount of normal leaf nucleoprotein, and that this did not release its nucleic acid on denaturation by heat. On ageing, however, the normal leaf nucleic acid would have been destroyed by the ribonuclease that accompanies it (Pirie, 1950), and so would no longer be present in the protein coagulum.

Fission by perchloric acid

Extraction with $HClO_4$ (Ogur & Rosen, 1950) is now becoming a standard method for separating the nucleic acids from tissues, and it is generally accepted that ribonucleic acid can be extracted in 24 hr. at 4° with N-$HClO_4$, whereas the deoxyribonucleic acid separates from the protein only after heating for 20 min. in 0·5 N-$HClO_4$ at 70°. With TMV the cold treatment releases only two-thirds of the nucleic acid, but it is all released on exposure to N acid at 20°. An experiment illustrating the release on heating and with $HClO_4$ is set out in Table 1. This shows clearly the complete or nearly complete separation of P from the protein except when the fresh preparation is heated. It also shows that the heated fluids are as suitable for measurement of ultraviolet absorption as the $HClO_4$ extracts, and that the amount of absorption is about the same under the two conditions. The density at 400 mμ. is included as an indication of the clarity of the extracts.

Material that absorbs at 260 mμ. and is not extracted by cold $HClO_4$ but is by hot, is commonly considered to be deoxyribonucleic acid (DNA). By this criterion that part of the nucleic acid which is not extracted from TMV in 24 hr. at 4° could be looked on as DNA, and the presence of small amounts has already been demonstrated in some preparations of TMV by more trustworthy methods (Hoff-Jørgensen, 1952; Holden & Pirie, 1955*b*). No DNA is, however, detectable by the Dische (1955) procedure in TMV preparations that have been incubated with citrate, under the conditions described below, and then recovered by ultracentrifuging. Though it is a common component of TMV preparations, DNA is not therefore essential for virus activity.

Recognition of extraneous ribonucleic acid

Young infected leaves of tobacco and other plants contain NP, and it can be isolated by methods similar to those generally used in the preparation of viruses by ultracentrifuging (Pirie, 1950). This will contaminate virus preparations, but the extent of contamination is diminished by many of the pretreatments given to leaves and leaf extracts because NP is less stable than the plant viruses generally investigated. Freezing, heating to 60°, addition of ethanol or phosphate, and precipitation by $(NH_4)_2SO_4$ or acid all modify it so much that it no longer resembles TMV, and all these treatments are used in different methods of preparing TMV.

When young infected tobacco leaves are the source of TMV, and when gentle isolation procedures are used so as to get evidence about the original state of the virus in the leaf, the amount of material with properties similar to NP in a pre-

Table 1. *Separation of nucleic acid from the protein of two types of TMV preparation*

Two 10 mg. lots of freshly prepared Rothamsted culture of TMV and two 10 mg. lots of the same preparation after it had been incubated with citrate and isolated again by ultracentrifuging were used. One of each pair was heated to 100° and the other extracted at 20° with $HClO_4$ as described in the text. Phosphorus determinations were made on the whole protein precipitate and on fractions of the supernatant fluids. Ultraviolet-light-absorption measurements were made in a 1 cm. cell on another fraction of each fluid diluted to be the equivalent of 18 ml. for the whole 10 mg. of virus. The control solutions were the appropriate citrate–NaCl mixture or $HClO_4$.

Type of preparation and method of extraction	Phosphorus (μg.) from 10 mg. in the		$E_{1cm.}$ of the fluid diluted to 0·555 g. of initial TMV/l.		
	Protein ppt.	Fluid	260 mμ.	280 mμ.	400 mμ.
Freshly prepared and then heated	5·2	56	1·40	0·77	0·04
Same lot extracted with $HClO_4$	0	61	1·46	0·94	0·015
Incubated with citrate, ultracentrifuged, and then heated	1	54	1·10	0·61	0·04
Same lot extracted with $HClO_4$	0	55	1·14	0·72	0·01

paration becomes large. The results given in the preceding section suggest that this is also true of preparations made in other Laboratories. So far there is no method for distinguishing between NP that is merely mixed with TMV and similar material combined with TMV. That question is discussed elsewhere (Pirie, 1949, 1953, 1956*a*); here discussion is about the determination of one form of P, which will be called labile P, in a TMV preparation and with the factors that affect the quantity of it.

When preparations that have been made by two cycles of differential ultracentrifuging by the method already outlined are ultracentrifuged again, at a concentration of about 7 g./l., from neutral water, the supernatant fluid may contain only 1 mg. of P/l. This is less than one-fortieth of the P sedimented, and on the repeated ultracentrifuging there is little further removal of unsedimentable P. The pellet can be resuspended easily in water or dilute buffer solution, and nothing can be sedimented from it in 10 min. at 10 000 *g*.

Preparations like this are comparable to those commonly called TMV. They are stable if protected from bacterial contamination and contain 0·5–0·6 % P, all of which is precipitated by 50 g. of TCA/l. Every preparation made in this way has contained material with many properties of NP. Outstanding among these is the ability to autolyse if incubated for a few hours, particularly with citrate ions, at 37° and in the presence of an antiseptic such as chloroform. When NP prepared from young uninfected leaves is incubated thus, most of the protein coagulates and the nucleic acid is degraded so far as to be no longer precipitated by TCA (Pirie, 1950). Citrate is more convenient for use here than phosphate, which Bawden & Pirie (1945) had used to bring about a similar action and also to aggregate TMV preparations, because the action can then be followed by total P determination. When the proportion of NP in a TMV preparation is small there is no visible coagulation of protein. The mechanism of this effect of citrate is not known, but TMV preparations made by ultracentrifuging contain 0·3–0·5 % of Ca, and Ca inhibits the action of leaf ribonuclease on NP (Pirie, unpublished work). It is therefore probable that citrate acts by sequestering the Ca.

For routine determination of the labile P in a virus preparation, an amount of the solution containing 3 mg. of dry matter is made up to 1 ml. in 0·02 M citrate buffer (pH 6·0) and incubated for 16–24 hr. at 37° in the presence of a drop of $CHCl_3$. It is cooled and 1 ml. of TCA (100 g./l.) is added; after standing for 5–10 min. on ice the precipitate is centrifuged down, the fluid poured off and total P determined on both fluid and precipitate. A few representative figures are collected in Table 2. Five

Table 2. *Amount of labile phosphorus in different types of TMV preparation*

Methods used for making the leaf extracts and separating the virus from them are described in the text.

Type and duration of infection	Breadth of leaf and type of extract	Method of preparation	Percentage of total P in the preparation	Percentage of total P that is labile
Rothamsted strain, 20. ix. 53 to 14. x. 53	<8 cm. frozen leaves minced	Ultracentrifuged twice	0·52	17
	Unfrozen leaves minced	Ultracentrifuged twice	0·80	41
	Fibre residue from last milled	Ultracentrifuged twice	0·74	29
Strain U2, 7. i. 55 to 14. ii. 55	<8 cm. fresh leaves minced	Ultracentrifuged twice	0·71	47
	Fresh leaves pressed through slot	Ultracentrifuged twice	0·50	22
Rothamsted strain, 7. iii. 55 to 31. iii. 55	<5 cm. minced	Ultracentrifuged twice	0·9	55
	>5 cm. still green, minced	Ultracentrifuged twice	0·81	45
	>5 cm. lower yellow, minced	Ultracentrifuged twice	0·71	38
	<5 cm. minced	Sap left 24 hr. at 16° before ultracentrifuging	0·56	37
Rothamsted strain, 31. iii. 55 to 2. v. 55	<8 cm. minced	Ultracentrifuged twice immediately	0·49	20
	<8 cm. minced	Ultracentrifuged after $(NH_4)_2SO_4$ precipitation	0·51	2
Datura strain, 30. vi. 55 to 15. viii. 55	8 cm. minced	Ultracentrifuged twice immediately	0·51	24
	8 cm. minced	Sap left 48 hr. at 21° before ultracentrifuging	0·47	13

different batches of plants were used, and each was subdivided into 2, 3 or 4 for comparison of different types of treatment. Several points stand out clearly: The three virus strains are essentially similar. There is material resembling NP in TMV preparations from yellow leaves, although very little NP can be made from uninfected yellow leaves (Pirie, 1950). Labile P can amount to half the total, but the analyses in Table 2, and all the others that have been made, show that this happens only when the total P content of the preparation is anomalously high. But one-quarter of the P in a preparation with the accepted P content (0·50–0·56 %) can be labile. The proportion of labile P in a preparation is diminished by most variations in the preparative technique. Thus freezing the leaves before mincing, freezing the sap, allowing the sap to age at room temperature before ultracentrifuging, and a single precipitation with $(NH_4)_2SO_4$ all remove labile P, and all are already known to destroy NP.

The effect of varying the technique used in grinding up the leaf is also of interest. Leaf blades are generally passed through a domestic meat mincer. An alternative in small-scale work is to force the leaf through a slot 5–25 μ. wide between a steel cylinder and plug. This requires a pressure of 1–2 tons/sq.cm. (Pirie, 1956*b*). Preparations can be made from the residues after mincing and pressing out the sap by grinding in a roller mill (Bawden & Pirie, 1944), or in a high-speed macerator, or by pressing through the slot. These variations diminish the amount of labile P, and preparations made from the fibre residues generally have only 10 % of their P in this form. TMV made from fibre residues is consistently less infective than that made from sap (Bawden & Pirie, 1956), but diminished infectivity is not an attribute of preparations that for other reasons contain little labile P. Infectivity is not, for example, significantly affected by incubation with citrate.

There is no reason to think that these methods of making TMV preparations with diminished NP content are all effective for the same reason, but there is good reason to think that an enzyme is acting during the incubation with citrate. Labile P is split off at about the same rate throughout the pH range 5·5–7·5 with citrate concentrations in the range 0·1–0·003 M. But it is not split off on simple contact with citrate, nor on prolonged exposure at 0°, nor on incubation in the presence of formaldehyde (5 g./l.). This much can be shown when solubility in TCA (50 g./l.) is used as a criterion of labile P. Exposure to citrate at 0° does, however, cause changes that can be recognized by ultracentrifuging. Thus a third ultracentrifuging from 0·02 M citrate increases the P content of the supernatant fluid three- to ten-fold, compared to the value in water, with a corresponding diminution in the labile P content of the pellet. But the P in the supernatant fluid is still precipitable by TCA if the temperature during the ultracentrifuging is kept low. From this precipitate ribonucleic acid is easily prepared by the method already described for the preparation from NP (Pirie, 1950). This is the main reason for looking on labile P as ribonucleic acid, but there is no evidence that all the labile P is in this form. The facts are, however, compatible with the idea that ribonuclease in the preparations is the effective agent in these changes, and in a later section it is shown to be present in the preparations.

These results agree in many respects with those of Siegel & Wildman (1954) and Ginoza, Atkinson & Wildman (1954). They were particularly interested in the association of coloured material with TMV preparations, in the dissociation of the complex when it is exposed to phosphate and citrate, and in differences in the abilities of different virus strains to associate with the colour, but they also observed the presence of some labile P. The amount of labile P was not stated, but it was presumably small because they were working with virus preparations made from leaves that had been through a colloid mill, and the extracts had been frozen.

Presence of extraneous protein

Preparations of TMV made by salt precipitation and ultracentrifuging still carry enough normal leaf protein to elicit an anaphylactic response in guinea pigs sensitized to normal protein. This ability can be removed by incubating heated preparations with trypsin (Bawden & Pirie, 1937*b*). The amount of normal protein in these preparations is probably small. If the labile P in TMV preparations is contributed by material like NP the amount of normal protein present will be much larger because NP consists mainly of protein. A few comparisons of the effect of incubating TMV with citrate and with citrate in the presence of trypsin have therefore been made.

The supernatant fluid from preparations that have been ultracentrifuged after incubation with citrate contains only 2·6–3·2 atoms of N for each atom of P. The reason for this discrepancy from what would be expected if the action were a simple and complete breakdown of nucleic acid has not been determined, but when trypsin (Armour 'crystalline') is also present during the incubation the amount of N in the supernatant is doubled. There is no reason to doubt that, during the incubation without trypsin, breakdown of nucleic acid is predominant and the protein of the NP accompanies the TMV into the pellet, but that it is split

during trypsin incubation. The results were similar when TCA precipitation was substituted for ultracentrifuging.

These TMV preparations appear to contain enough ribonuclease to bring about complete fission of the NP when they are incubated with citrate, for no more P becomes unsedimentable when they are incubated with trypsin or with the other commercial enzyme mixtures that have been tried. Preparations made from sap aged at room temperature or by precipitation with $(NH_4)_2SO_4$ contain only 2% or less of their N in a form that does not sediment after incubation with trypsin.

Precipitation of TMV by ammonium acetate

The number of salts suitable for precipitating most proteins from aqueous solution is limited, and $(NH_4)_2SO_4$ is used more commonly than any other. It is also generally used with TMV, but there is no reason for the restriction because TMV is precipitated reversibly by a wide range of substances. Best (1940) found that the precipitate separating from ammonium acetate solution under critical conditions, like the precipitates brought out by nicotine and arginine (Bawden & Pirie, 1940), dispersed completely when the solution was shaken, and re-formed again slowly. Because the ammonium ion is particularly effective at displacing other cations from colloidal systems, and because ammonium acetate evaporates easily from a preparation *in vacuo*, the conditions for precipitation by ammonium acetate have been examined more carefully than those for the other salts, and differences between virus strains and between virus preparations in different states have been found.

With a solution of a fresh preparation of the Rothamsted type strain of TMV isolated by ultracentrifuging only and containing 5 g. of TMV/l. in 0·01 M sodium citrate (pH 6) a shimmer is immediately apparent when enough ammonium acetate solution has been added to make the solution 1·3 M. At 1·6 M concentration the shimmer slowly increases to an opacity which disappears if the solution is shaken briskly. It reappears if the solution is left quite still for a few minutes, but appears more quickly if the solution is gently agitated. Even after many hours, precipitation is incomplete at 1·6 M. A 2 M concentration gives complete precipitation, but the intermediate stages cannot then be completely dispersed by shaking.

The phenomena are similar, but more sharply demonstrable, with part of the same preparation of TMV after incubation at 37° for 16 hr. in 0·01 M citrate (pH 6) in the presence of $CHCl_3$, or with a preparation isolated from the infected sap by precipitation with $(NH_4)_2SO_4$ and at pH 3·3 (Bawden & Pirie, 1943). The shimmer is then apparent with 0·8 M ammonium acetate and precipitation is, after a time, apparently complete with M ammonium acetate. More prolonged incubation, or incubation with the mixture of enzymes in commercial trypsin, does not lead to a virus preparation that begins to be precipitated at a lower concentration than 0·8 M, but the precipitates formed are perceptibly more compact and less easily dispersed.

Under the same conditions the *Datura* strain of TMV is less easy to precipitate. Even at 2·5 M ammonium acetate the precipitation of unincubated preparations is incomplete, and with incubated preparations 1·3 M concentration is needed for precipitation. These precipitates also disperse on shaking, and the re-formation is hastened by gentle shaking.

Other salts, including $(NH_4)_2SO_4$, illustrate the same phenomena, but less dramatically. Conditions have not been found in which a properly flocculated precipitate can be dispersed, but if a partially precipitated mixture is shaken vigorously and then quickly centrifuged for 5 min. at 600 *g* the initially clear supernatant will again be partially precipitated on standing. The fact that the amount of precipitate in a system, or even the very existence of two phases in it, is so greatly affected by small rates of shear goes far to explain the difficulty often experienced in attempts to centrifuge a precipitate that appears to be settling well under gravity (cf. Pirie, 1955). The phenomenon may also be in part responsible for the scatter of the results of Loring (1940) for the 'solubility' of TMV in $(NH_4)_2SO_4$ solutions of varying concentration.

This type of phenomenon is, however, mainly of interest because it offers another possibility for following the progressive changes that go on in virus preparations during storage. In this respect changes in precipitability by certain salts under critical conditions are comparable with the change from 'flagellar' to 'somatic' type of precipitation with antiserum (Bawden & Pirie, 1945). The properties of these materials are of interest in all the forms in which they can exist, but it is the properties exhibited before *in vitro* changes have taken place that are most relevant to the biological properties of viruses.

Ribonuclease activity

The changes that take place in TMV preparations when incubated in citrate suggest that leaf ribonuclease (LRNase) is present, and a series of measurements of the LRNase activity of different types of preparation has been made. The activities are always rather low and the material that is being tested as an enzyme contains nucleic acid, part of which is undergoing fission. This introduces some difficulty in interpreting the results, but in most experiments the amount of labile P added along

with the material being tested can be kept below 6 % of the P present in the yeast nucleic acid substrate. This is, for example, so in all but one of the tests set out in Tables 3 and 4.

At first the conditions defined by Holden & Pirie (1955*a*) for the assay of LRNase were used, but to get greater sensitivity the concentration of substrate yeast nucleic acid was later lowered from 400 mg. to 50 mg. of P/l. and the duration of incubation was increased to 24 hr. The experiments quoted were carried out under these conditions. Unstoppered tubes or tubes with glass or polythene stoppers were used because rubber stoppers are one cause of erratic results. Holden & Pirie found that zinc was an exceptionally powerful inhibitor of LRNase, and the zinc oxide used as a filler in many types of rubber probably dissolves slowly.

Differences between preparations of TMV. It is clear from Table 3 that the LRNase activity of a TMV preparation diminishes as the sap ages, and is greatly diminished by precipitation with $(NH_4)_2SO_4$. Preparations made from the fibre after pressing out the leaf sap have also a low activity. The presence of LRNase in a preparation therefore parallels the presence in it of the labile P discussed above, and this increases the probability that the labile P comes from NP associated with the TMV, for NP carries LRNase with it (Pirie, 1950). The precision of the enzyme assays is too small to allow a strict correlation with labile P content, but more than 100 preparations have been assayed for LRNase and labile P without a gross departure from parallelism being found. The effect of citrate incubation on the enzyme activity is therefore of

Table 3. *Comparison of the LRNase activities of preparations of the Rothamsted strain of TMV made in different ways*

Methods used for making the virus preparations and for determining labile P are given in the text. For the LRNase assay the indicated amount of material to be tested was mixed in 2 ml. final volume of 0·01 M citrate (pH 6) containing yeast nucleic acid (50 mg. of P/l.) and a little thymol. Samples (0·5 ml.) were withdrawn at the start and after 5 and 24 hr. incubation at 37°, and pptd. with the uranyl nitrate and TCA (UrTCA) reagent (Holden & Pirie, 1955*c*). Phosphorus was determined on the supernatant. To get strictly comparable figures it is necessary to subtract half of the figure in the labile P column from the 24 hr. figure because that amount of P/ml. is not contributed by the substrate YNA.

Source of preparation	Amount added (mg.)	Amount of P in prep. (μg.)	Amount of labile P in 2 ml. (μg.)	P soluble in UrTCA, in mg./l. after 0 hr.	5 hr.	24 hr.
Fresh sap	0·7	5	1·5	1	13	46
One-day-old sap	0·7	4	1·0	1	10	34
Extract from finely ground washed fibre	0·7	4	0·8	1	5	13
	2·3	13	2·6	1	7	31
Ammonium sulphate ppt. from sap	3	14	0	1	2	11

Table 4. *A comparison of the LRNase content of supernatants and pellets after ultracentrifuging TMV treated in various ways*

Four 64 mg. lots of the Rothamsted strain of TMV purified by two cycles of ultracentrifuging were recentrifuged after the treatments specified. The volume of fluid in each tube was 7·7 ml.; after the run 5 ml. was pipetted off, and the remainder of the fluid was poured off. The drained pellets were suspended and samples analysed for dry matter, total P, labile P and RNase activity. The figure put down for 'labile P' on the four supernatant fluids is the amount of P not precipitable by uranyl nitrate and TCA (UrTCA) after incubation but, unlike labile P proper, much of it was already unprecipitable as a result of the earlier incubation. To get comparable figures, half of this value should, however, be subtracted from the mg. of P/l. value at 24 hr. LRNase assay as in Table 3.

Treatment before the third ultracentrifuging	Quantity of supernatant (s) or pellet (p) tested	Weight of P in the material tested: Total (μg.)	Labile (μg.)	P in mg./l. soluble in UrTCA after different times of incubation: 0 hr.	5 hr.	24 hr.
In water at 0°	0·5 ml. (s)	1·5	(1·5)	1	11	44
	1·7 mg. (p)	13	5	1	16	51
In water for 16 hr. at 37°	0·3 ml. (s)	15	(15)	8	18	33
	1·7 mg. (p)	9	3	1	5·6	28
In 3 ml. of 0·02 M citrate (pH 6·0), for 16 hr. at 37°, then diluted to 7·7 ml.	0·1 ml. (s)	6	(6)	4	10	28
	1·7 mg. (p)	9	0	1	2	7
	9·0 mg. (p)	47	1	2	6	37
Citrate added at 0° immediately before ultracentrifuging	0·1 ml. (s)	1·5	(1·5)	3	10	46
	1·7 mg. (p)	12	4	1	5·2	22

interest, and an experiment is set out in Table 4. It shows that TMV sedimented from citrate has a diminished LRNase activity compared to TMV sedimented from water, and that a period of incubation causes still greater diminution. There is also a diminution after incubation in water but it is not so great. Table 4 also shows that much of the enzyme activity that disappears from the TMV pellet as a result of these treatments is present in the supernatant fluid. A balance sheet cannot be drawn up because LRNase shows an enhanced activity in the presence of TMV; this is discussed below. A comparison between tests 1 and 4 makes it clear that contact with citrate at 0°, which has already been shown to make much of the labile P unsedimentable, is sufficient to increase the enzyme activity of the supernatant and diminish that of the pellet. Test 3 (Table 4) shows that the activities of both fractions are diminished by incubation, and this is confirmed in separate tests with TMV incubated for various periods. The instability of LRNase on incubation at 37° has already been commented on (Holden & Pirie, 1955*c*). Experiments of this type cannot be interpreted in detail because of the conflicting effects of enzyme destruction and activation; but, during many repetitions with different incubation procedures, conditions have not been found in which the loss of enzyme activity by the pellet has not been accompanied by loss of labile P and increased ease of precipitation by ammonium acetate.

When TMV preparations, made by ultracentrifuging only, are subjected to further cycles of ultracentrifuging there is always a layer of sedimented but uncompacted material lying over the pellet in the centrifuge tube. This material has a higher content of labile P and LRNase than the pellet but, although sedimenting more slowly than the TMV pellet, it contains material related to TMV and it is generally only slightly less infective and serologically active than the pellet underlying it (Bawden & Pirie, 1956). The amount of this material is greatly diminished by any of the treatments—heating, exposure to dilute phosphate or citrate, precipitation by acid or $(NH_4)_2SO_4$ etc.—that aggregate TMV (Bawden & Pirie, 1945). By ultracentrifuging at only 30 000 *g* the proportion of the material remaining uncompacted is increased and the LRNase content of the pellet correspondingly diminished. Although the uncompacted material contains TMV, its physical and enzymic properties resemble closely those of a fraction that can be made by similar methods from NP preparations from uninfected plants. The attempt to systematize the effects of varying both the sequence of sedimentations and the ionic environment will be dealt with in a later paper on NP.

Effects of TMV on LRNase activity

So far it has not proved possible to make an infective TMV preparation showing no LRNase activity. Activities even lower than that shown by preparation 3 in Table 4 can be made by $(NH_4)_2SO_4$ precipitation and repeating the ultracentrifuging, this time from 0·05 M ammonium acetate containing 2 g. of yeast nucleic acid/l. Centrifuging in this way from yeast nucleic acid is also effective in removing LRNase from NP (Pirie, unpublished observations). LRNase is very easy to detect in the presence of TMV, for the virus has an activating or protective effect. Thus in tests similar to those in Table 4, 5 mg. of a TMV preparation that had been sedimented from YNA made 2 mg. of P/l. soluble in 5 hr. and 5 mg. in 24 hr., whereas with 0·03 unit (Holden & Pirie, 1955*a*) of pea-seedling LRNase the figures were 3 and 7 mg. of P/l. at these times. With TMV and pea LRNase together, however, they were 29 and 38 mg. of P/l. again at the same times. It is unlikely that this effect is caused by the TMV protecting the enzyme from destruction because it is also shown in brief experiments and in experiments at 25°. It is more probable that it is an example of the promotion of enzyme–substrate complex formation by a third substance, and is therefore similar to the activation of cellulase by inert protein discussed by Whitaker (1952).

TMV that has been precipitated with TCA gives another example of what is probably a similar activation. Equal volumes of the solutions, 10 g. of TMV/l. and 100 g. of TCA/l., are mixed and left for 5–15 min. at room temperature. The precipitate is centrifuged down hard, washed once with water, suspended, and neutralized with dilute KOH; nearly all the P of the original TMV is still present. If a sample of the neutral suspension is immediately reprecipitated with TCA the P is reprecipitated, but if the suspension is incubated the P becomes unprecipitable to an extent depending on the components of the incubation mixture. A few results are set out in Table 5, and from this it is clear that, as in the action of LRNase on other substrates, the presence of ions is needed for the action, and citrate ions are among the more effective. It is also clear that the action is inhibited by formaldehyde and goes faster in the presence of very small amounts of LRNase. After a second precipitation with TCA there is no action.

There is therefore no reason to look on this action as anything but an unusually sensitive test for LRNase; it is probably not akin to the nucleic acid fission that takes place in TMV treated with 0·033 N-NaOH (Grégoire, 1950). The sensitivity may be the result of intimate association of enzyme and substrate in the coagulum, but it is at least partly the result of the use of TCA as precipitant.

Table 5. *Effect of incubating TMV that has been precipitated with TCA*

0·1 ml. lots of a suspension of TMV (20 g./l., pH 6) that had been precipitated with TCA were diluted to 1 ml. in the manner stated, and incubated with a drop of $CHCl_3$ for the time stated. TCA solution (1 ml. of 100 g. of TCA/l.) was added and P determined on both fluid and precipitate.

Suspending fluid	Temp.	Percentage of P soluble in TCA (50 g./l.) after incubation for 5 hr.	24 hr.	48 hr.
Water	35°	0	30	45
0·005 M citrate (pH 6)	35°	25	45	—
0·02 M citrate (pH 6)	35°	35	70	90
0·02 M citrate (pH 6)	0°	0	0	10
0·02 M citrate (pH 6) +0·01 unit of LRNase	35°	80	90	—
0·02 M citrate (pH 6) +5 g. of formaldehyde/l.	35°	0	5	10
0·02 M ammonium acetate	35°	20	50	—
0·1 M ammonium acetate	35°	30	60	—
0·1 M-NaCl	35°	15	45	—
0·003 M ethylenediaminetetraacetate	35°	20	45	—

TCA precipitates nucleic acid incompletely from dilute solution (Holden & Pirie, 1955*c*), but in these experiments, presumably because the nucleic acid is being carried down along with the TMV protein, TCA is satisfactory. When it is replaced by the uranyl nitrate and TCA reagent there is more complete precipitation of nucleic acid fission products; hydrolysis is then shown only after more prolonged incubation and with some preparations of TMV it remains incomplete. This suggests that, as in other actions where many links of the same type are being hydrolysed enzymically, the first stages of the action are disproportionately easy to detect. Preparations of TMV that have been purified so far that the hydrolysis of yeast nucleic acid by them is only just perceptible do not have their enzymic activity against yeast nucleic acid enhanced by precipitation by TCA. There is, therefore, no reason to think that nuclease is being produced here from an inactive precursor.

DISCUSSION

From these observations it is clear that preparations of tobacco mosaic virus made by quick and gentle methods contain material that is not present in preparations that have been made by methods that are rougher or more dilatory. There is no conclusive evidence as yet that these other materials are combined with the virus and not simply mixed with it, and it is probable that it would be wisest to regard many of the preparations described here as contaminated with adventitious components of the leaf not necessarily associated with the virus. This is a question that cannot be settled by the technique used here, and the only relevant evidence is that preparations made from leaves so old that little or no nucleoprotein would have been isolated had they been uninfected, contain a significant amount of material with an instability and enzyme activity similar to that of normal leaf nucleoprotein (NP). The fact that tobacco mosaic virus made from leaves that have been frozen or very finely ground in a roller mill or by pressing through a narrow slot contains less labile phosphorus does not give positive support to either point of view. Freezing destroys much of the NP, but fine grinding does not. The interpretation of this particular piece of evidence is complicated by the fact (Bawden & Pirie, 1956) that tobacco mosaic virus made by fine grinding is invariably less infective than that made normally.

Whether or not it is adventitious, material similar to NP is present in tobacco mosaic virus preparations made by the method used here, and it is probably present in preparations made by the methods used in many other Laboratories. Its recognition is therefore a matter of importance, and a few tests may be proposed that should be applied to every tobacco mosaic virus preparation destined for a study of amino acid, purine, or pyrimidine composition or for the measurement of any physical properties. After incubation with citrate and chloroform the amount of phosphorus and nitrogen appearing in forms not precipitable by trichloroacetic acid should be negligible, otherwise it is reasonable to conclude that material like NP is present. On incubation with trypsin no nitrogen should become soluble in trichloroacetic acid except the 0·5% that would come from threonine split off if enzymes able to catalyse the action studied by Harris & Knight (1955) are present.

When freed from these materials, preparations of tobacco mosaic virus appear to contain only the cations needed to maintain neutrality, and protein and nucleic acid bonded in such a manner that they resist attack by those nucleases and proteases that have been tested. This is the limit beyond which fractionation has not been carried without loss of

infectivity. To avoid ambiguity, preparations in this state need a designation, and this should distinguish them from those in general use without raising the issue of whether the removal of all the other materials from the preparation can properly be called a purification (cf. Pirie, 1949, 1953). The term TMV (*L*) is suitable because of the limiting character of this type of preparation.

Experience with the strains of tobacco mosaic virus studied here suggests that by the time fractionation has been carried so far as to have removed these extraneous forms of phosphorus and nitrogen, the virus will no longer be in its original state. There is an increase in the readiness with which tobacco mosaic virus shows anisotropy of flow; this has been commented on repeatedly, but elaborate measurements would be needed to put it on an objective basis. Two other properties are easier to define. The behaviour on precipitation with ammonium acetate is described here, and it is probable that any tobacco mosaic virus preparation that does not become precipitable at a lower concentration after incubation with citrate and chloroform, or trypsin, has already been modified during the process of purification. The criterion for native tobacco mosaic virus has to be put in this slightly roundabout way because the different strains of virus precipitate with different concentrations of ammonium acetate; it is the change in precipitability that is significant. Parallel with this change is the change in the concentration at which a neutral and relatively salt-free solution of tobacco mosaic virus deposits a liquid-crystalline layer. It is unusual to find a fresh preparation, made by ultracentrifuging only, that will deposit this layer even when as concentrated as 60 g./l., but as *in vitro* changes proceed, liquid-crystallization becomes easier. Preparations that have been ultracentrifuged after being incubated with citrate generally form the layer at 20–25 g./l., and when they have matured further they form it at even lower concentrations. The limit found with the preparations studied here was 6·8 g./l.

It is tempting to try to simplify the picture and interpret all these phenomena in terms of two variables: the extent to which tobacco mosaic virus is contaminated in a purely mechanical way by extraneous substances, and the extent to which a hypothetical fundamental unit, perhaps 300 mμ. long, becomes broken or aggregated linearly. These attempts may yet succeed and it may be a coincidence that the treatments which remove NP from a preparation are those that promote aggregation. But it is also possible that the removal of material like NP from the ends of the tobacco mosaic virus rods is an essential preliminary to aggregation.

SUMMARY

1. Part of the phosphorus in many tobacco mosaic virus preparations becomes soluble in trichloroacetic acid when the preparations are incubated. There is evidence that this is caused by the fission of normal leaf nucleoprotein by ribonuclease.

2. Some steps in the purification remove the normal nucleoprotein but other properties of the virus, notably its precipitability by ammonium acetate, are altered at the same time.

3. Plant ribonuclease is more difficult to remove and it is detectable in all preparations. The smallest amount is found in those that have been ultracentrifuged from salt solutions in the presence of nucleic acid. Tobacco mosaic virus that has been denatured by trichloroacetic acid is a particularly sensitive system for detecting this residual nuclease.

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Studies on the Metabolism of Mammary Tissue *in vitro*

1 OXIDATION OF ACETATE AND GLUCOSE BY SLICES OF LACTATING RAT MAMMARY GLAND

By W. G. DUNCOMBE and R. F. GLASCOCK
National Institute for Research in Dairying, University of Reading

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Respiratory experiments carried out in this Institute have indicated that non-ruminants, unlike ruminants, use both glucose and acetate for fatty acid synthesis in the mammary gland. Thus Folley & French (1949, 1950) found that the respiratory quotient (R.Q.) of mammary slices from both rats and rabbits was greater than unity (about 1·5) in media containing glucose as the only substrate. When acetate was the only substrate the R.Q. was consistently less than unity (about 0·85), but it increased to a value above unity (about 1·3) when glucose was added, and acetate was then found to disappear from the medium. These results were interpreted as indicating that both acetate and glucose can function as sources of carbon for fatty acid synthesis in the mammary gland of non-ruminants; but because acetate is only very slowly oxidized it can be used for fatty acid synthesis only when carbohydrate is present as a source of energy.

This work was extended by later experiments with the isotope-incorporation technique. It was found (Balmain, Folley & Glascock, 1952) that rat mammary slices incorporate considerable amounts of ^{14}C from [*carboxy*-^{14}C]acetate into fatty acids in the presence of glucose but virtually none in its absence. In later work (Balmain, Folley & Glascock, 1954) it was found that acetate and glucose carbon are used about equally for fatty acid synthesis when both substrates are present together.

If a single substrate only is available the manometric technique indicates whether or not it is oxidized. When more than one substrate is present, however, no means is available for determining the relative amounts of each oxidized. This is obviously a question which can be answered best by the use of labelled substrates, but is one which was not studied in the work cited (Balmain *et al.* 1952, 1954), in which only the incorporation of substrates into mammary-gland fatty acids of rats and sheep was measured. The current experiments have therefore been undertaken to discover the source of respiratory carbon dioxide and to determine the effect, if any, of one substrate on the oxidation of another. It is thus hoped to throw light on the sources of energy and metabolic pathways in the mammary gland. Some of this work has already been communicated in preliminary form (Duncombe & Glascock, 1954). Since then papers by Hirsch, Baruch & Chaikoff (1954) and by Abraham, Hirsch & Chaikoff (1954) have appeared. Where they duplicate our work these investigators largely confirm our findings.

EXPERIMENTAL

Animals. The rats used were hooded Norway rats, killed by breaking their necks at the 13–15th day of their first lactation. Mammary glands were dissected out and sliced with a hand microtome. When sliced tissue from more than one rat was required, the slices were pooled.

Batches (0·5 g.) of slices were incubated in bicarbonate–saline (Krebs & Henseleit, 1932), to which the necessary substrates had been added. The reaction vessel was a 50 ml. round-bottomed flask, closed with a tall, hollow-glass head (B.24 standard joint). A stopcock, joined to a B.10 standard socket, was sealed to the top of the head so that the vessel could be attached by the socket to a vacuum manifold. A gas-inlet tube with a stopcock was sealed into the side of the head, and reached nearly to the bottom of the flask when the head was inserted. The gas phase was $O_2 + CO_2$ (95:5), which was passed in through the side arm and run to waste through the B.10 standard joint until the air in the unit had all been displaced. This procedure was carried out on a manifold while the unit was immersed in the water bath at 37°. The stopcocks were closed and incubation with shaking then proceeded for 3 hr. At the end of that time the units were removed from the water bath, frozen in a solid CO_2–acetone bath and attached one at a time to a high-vacuum apparatus. Acid was introduced through the side arm, and the contents of the flask were melted; CO_2 in the unit was separated from the oxygen by pumping slowly through liquid-air-cooled traps and measured in a calibrated manometric chamber. The specific activity of the CO_2 was found by counting measured portions in a gas counter. A portion was set aside for ^{13}C assay when this isotope was also present. The whole technique has been described in detail by Glascock (1954).

Substrates. The substrates used were uniformly ^{14}C-labelled and 1-^{14}C-labelled glucose and a mixture equivalent to doubly labelled sodium acetate ($^{14}CH_3.^{13}CO_2Na$), prepared by mixing together suitable amounts of the singly labelled salts. The concentrations of the substrates in the media were: glucose, 300 mg./100 ml.; sodium acetate, 0·02 M; sodium pyruvate, 0·02 M. Stock supplies of the labelled substrates were prepared by adding the calculated

activity to a weighed amount of the solid, dissolving both in water and evaporating to dryness. The specific activity of the CO_2 obtained by combustion of substrates was determined with the same gas counter as was used for the measurement of the specific activity of the CO_2 produced in the experiment. Sodium acetate was converted into silver acetate before combustion; glucose was combusted directly.

Coenzyme. The diphosphopyridine nucleotide (DPN) used in Expts. 62 and 63 was 95–100% pure and was obtained from L. Light and Co. Ltd.

RESULTS AND DISCUSSION

The rate of substrate oxidation is reported here as μg. of carbon/mg. dry wt. of tissue/hr. and is designated in the tables by the symbol q with the appropriate subscripts. Thus q_{CO_2H} and q_{Me} refer to the rates of oxidation of the carboxyl and methyl carbon atoms of acetate; q_{Ac} to the rate of oxidation of total acetate carbon (so that $q_{Ac} = q_{CO_2H} + q_{Me}$); q_{Gt} refers to the rate of oxidation of glucose total carbon (as measured by the rate of formation of labelled CO_2 from uniformly labelled glucose), and $q_{G\text{-}1}$ the rate of oxidation of the C-1 atom of glucose. The measurements reported therefore refer to added labelled substrates and do not necessarily reflect rates of oxidation of chemically similar endogenous substrates.

Oxidation of acetate

Effect of glucose. Table 1 shows the rate of oxidation of the two carbon atoms of acetate in the presence and absence of glucose. When acetate is the only substrate the ratio of the rate of oxidation of the carboxyl atom to that of the methyl atom is always close to the mean value of 1·42, although the actual rates of oxidation of the two carbon atoms vary widely. In all experiments the addition of glucose to the medium caused a decrease in the rates of oxidation of the two carbon atoms and an increase in the value of the ratio q_{CO_2H}/q_{Me}, indicating that there had been a greater decrease in the rate of oxidation of the methyl carbon atom than in that of the carboxyl carbon atom. In the table are also shown the mean values for q_{Ac} which are 0·273 in the absence of glucose and 0·144 in its presence. It is clear that the decrease in the rate of oxidation of acetate carbon cannot be due solely to dilution of labelled C_2 units arising from acetate by unlabelled C_2 units arising from glucose, because the rates of oxidation of the two carbon atoms are affected unequally.

The explanation put forward by Strisower, Kohler & Chaikoff (1952) to account for the different rates of oxidation of the two acetate carbon atoms by liver slices might apply also to these results. Briefly, their theory is that if equal activities of methyl- or carboxyl-labelled acetate enter the tricarboxylic acid cycle at each turn then, after equilibrium has been reached, the distribution of isotope in cycle intermediates will depend on the degree of dilution by unlabelled molecules entering the cycle. Taking the degree of isotope dilution as x

Table 1. *Effect of glucose on acetate oxidation by lactating-rat mammary tissue*

For experimental conditions see text. Rates of oxidation of the carboxyl and methyl carbon atoms of acetate (q_{CO_2H} and q_{Me}) are expressed as μg. of carbon/mg. dry wt. of tissue/hr.

	Glucose absent			Glucose present		
Expt. no.	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$
39	0·080	0·052	1·54	0·058	0·025	2·32
	0·069	0·045	1·54	0·064	0·029	2·20
41	0·119	0·083	1·44	0·081	0·036	2·12
	0·147	0·102	1·47	0·096	0·043	1·76
	0·123	0·084	1·48	0·102	0·058	2·23
	0·157	0·106	1·43	0·089	0·042	2·25
42	0·141	0·101	1·40	0·082	0·028	2·93
	0·176	0·124	1·42	0·082	0·038	2·16
	0·181	0·128	1·41	0·131	0·062	2·11
	0·132	0·098	1·35	0·086	0·039	2·20
43	0·180	0·122	1·47	0·099	0·049	2·02
	0·164	0·113	1·45	0·116	0·060	1·93
	0·177	0·130	1·36	0·101	0·051	1·98
	*	0·101	*	0·099	0·045	2·20
50	0·224	0·168	1·33	0·104	0·053	1·97
	0·258	0·203	1·27	0·130	0·066	1·97
	0·230	0·169	1·36	0·138	0·080	1·72
Mean	**0·160**	**0·113**	**1·42**	**0·097**	**0·047**	2·12
Mean q_{Ac}		**0·273**			**0·144**	
% isotope dilution, x		17·3			35·9	

* Sample lost.

(defined as meaning that after dilution x % of the mixture has been derived from unlabelled molecules) they deduce that in the carbon dioxide produced the ratio of isotope derived from carboxyl-labelled acetate to that derived from methyl-labelled acetate will be given by

$$\frac{q_{CO_2H}}{q_{Me}} = \frac{100+x}{100-x},$$

or, rearranging,

$$x = \frac{(q_{CO_2H}/q_{Me} - 1)}{(q_{CO_2H}/q_{Me} + 1)} \times 100, \qquad (1)$$

where q_{CO_2H}/q_{Me} is the quantity calculated by us in Tables 1 to 4 and which Strisower *et al.* (1952) call the CO_2 ratio. Thus the greater the degree of isotope dilution within the cycle the greater the value of this ratio.

By applying equation 1 to the data given in Table 1 where q_{CO_2H}/q_{Me} has the average values of 1·42 when acetate is the only substrate and 2·12 when glucose is added, it is found that the corresponding values of x are 17·3 and 35·9. This means that dilution by some endogenously derived substance entering the cycle amounts to 17·3 % when acetate is the only substrate and rises to 35·9 % when glucose is added.

If the theory of Strisower *et al.* (1952) is valid and there is, therefore, some endogenous diluent, its identity can at present be only guessed at. Washed mammary slices of the rat are known to contain very little available carbohydrate because the low R.Q. and negligible rate of incorporation of labelled acetate into fatty acids are both very much increased by the addition of glucose to the medium. It thus seems unlikely that the endogenously derived diluent entering the cycle is derived from carbohydrate, and three main possibilities for its origin remain, all of them connected with protein metabolism. It could be (1) α-oxoglutarate derived from glutamic acid, (2) oxaloacetate derived from aspartic acid, or (3) oxaloacetate derived from one of the many glucogenic amino acids, e.g. alanine.

Effect of pyruvate. The increase in the calculated degree of dilution occurring when glucose is added could be due to the formation of oxaloacetate by β-carboxylation of pyruvate formed by the glycolytic reactions. It was thus of interest to ascertain the effect of pyruvate itself on acetate oxidation. The results are shown in Table 2. Like glucose, pyruvate increased the value of the ratio q_{CO_2H}/q_{Me}, x being found to have the value 21·3 in the absence of pyruvate and 31·8 in its presence. In this respect pyruvate and glucose are therefore similar, both appearing to bring about the same degree of dilution in the tricarboxylic acid cycle. They differ, however, in their effects on the rate of oxidation of total acetate (q_{Ac}). Whereas glucose (Table 1) reduced the rate of acetate oxidation to about half, pyruvate (Table 2) had no consistent effect. In fact it will be noticed that whereas the addition of pyruvate nearly always reduced the rate of oxidation of the methyl carbon atom of acetate, it sometimes reduced and sometimes increased the rate of oxidation of the carboxyl carbon atom. The mean result over eleven different measurements with tissue from three animals was that the rate of oxidation of acetate total carbon was not appreciably changed by the addition of pyruvate.

Thus pyruvate brings about an increase in the q_{CO_2H}/q_{Me} ratio without at the same time reducing the rate of oxidation of acetate. This is contrary to the theory of Strisower *et al.* (1952), because it follows from their equations [from which equation

Table 2. *Effect of pyruvate on acetate oxidation by lactating-rat mammary tissue*

For details see text and Table 1.

Expt. no.	Pyruvate absent			Pyruvate present		
	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}	q_{CO_2H}	q_{Me}	q_{CO_2H}/q_{Me}
47	0·106	0·077	1·38	0·117	0·058	2·02
	0·099	0·068	1·46	0·123	0·069	1·79
	0·076	0·052	1·46	0·128	0·073	1·75
	0·146	0·093	1·57	0·120	0·065	1·85
55	0·182	0·125	1·46	0·195	0·118	1·65
	0·122	0·086	1·42	0·177	0·104	1·70
	0·200	0·144	1·39	0·195	0·115	1·70
72	0·067	0·037	1·81	0·129	0·057	2·26
	0·116	0·073	1·59	0·108	0·053	2·04
	0·091	0·055	1·66	0·107	0·046	2·32
	0·069	0·040	1·73	0·114	0·052	2·19
Mean	**0·116**	**0·080**	**1·54**	**0·134**	**0·074**	**1·93**
Mean q_{Ac}		**0·196**			**0·208**	
% isotope dilution, x		21·3			31·8	

(1) in this paper is derived], that an increase in the value of x must be accompanied by a decrease in the value of q_{Ac}. Thus their theory would appear to be not wholly valid or, superimposed upon the dilution effect that they consider, there must be also another effect when pyruvate is added: this would be to increase the rate of oxidation of total acetate carbon, leaving only the changed q_{CO_2H}/q_{Me} ratio as evidence that dilution in the tricarboxylic acid cycle has occurred at the same time.

Glucose, however, as already noted, causes an increase in the degree of dilution (x) which is accompanied by the expected decrease in the rate of acetate oxidation (q_{Ac}). Since glycolysis is active in rat mammary tissue (Terner, 1952), glucose must give rise to pyruvate. Hence, if as suggested above, pyruvate increases the rate of acetate oxidation, then glucose must have an over-riding inhibiting effect. One way by which such an inhibition could work is through competition between the reactions of the tricarboxylic acid cycle and those of the glycolytic pathway for some essential factor.

Effect of DPN and of nicotinamide. An essential factor common to both the tricarboxylic acid cycle and the glycolytic pathway is DPN. This coenzyme is essential for the oxidative decarboxylation of α-oxoglutaric acid to succinic acid and for the conversion, by malic dehydrogenase, of malic acid into oxaloacetic acid (Snell, 1953). In the glycolytic pathway it is essential for the oxidation of diphosphoglyceraldehyde to phosphoglyceric acid and for the oxidative decarboxylation of pyruvate to acetate.

If the reduction in the rate of acetate oxidation consequent on the addition of glucose is due to competition for DPN, then the addition of DPN to a preparation metabolizing acetate and glucose should increase the rate of oxidation of acetate. Actual experiments, however, were negative or inconclusive, as shown in Table 3. The small increase in the rate of oxidation of acetate (where agreement between replicates was poor) was not confirmed in Expt. 63, and in any case would be too small to compensate for the reduction in the rate of acetate oxidation (nearly 50%) known to be caused by glucose (Table 1). These negative results are not of course conclusive: the coenzyme may, in these preparations, have failed to penetrate the cells or may have been destroyed by nucleotidases. The first possibility calls for experiments with cell-free preparations (which have not yet been carried out), but the second could be investigated to some extent by the use of nicotinamide. This compound

Table 3. *Effect of DPN on the rate of oxidation of acetate carbon by lactating-rat mammary-gland slices metabolizing acetate and glucose together*

Concentration of DPN used: $2{\cdot}5 \times 10^{-4}$M in Expt. 62 and 10^{-3}M in Expt. 63. For details see text and Table 1.

Expt. no.	DPN absent		DPN present	
	q_{CO_2H}	q_{Me}	q_{CO_2H}	q_{Me}
62	0·062	0·033	0·097	0·046
	0·070	0·041	0·093	0·042
	*	*	0·078	0·038
Mean	**0·066**	**0·037**	**0·089**	**0·042**
63†	—	0·026	—	0·026
	—	0·032	—	0·023
	—	0·033	—	0·023
	—	0·033	—	0·032
Mean	—	**0·031**	—	**0·026**

* Sample lost. † With [Me-^{14}C]acetate only.

Table 4. *Effect of nicotinamide on acetate oxidation by lactating-rat mammary tissue in the presence of glucose*

Concentration of nicotinamide used was 0·02M. For details see text and Table 1.

Expt. no.	Nicotinamide absent			Nicotinamide present		
	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$
62	0·062	0·033	1·88	0·142	0·079	1·80
	0·070	0·041	1·71	0·118	0·061	1·93
	*	*	*	0·135	0·072	1·88
Mean	**0·066**	**0·037**	**1·79**	**0·132**	**0·071**	**1·87**
Mean q_{Ac}		**0·103**			**0·203**	
85	0·106	0·060	1·76	0·165	0·101	1·63
	0·162	0·095	1·71	0·226	0·140	1·61
	0·116	0·068	1·71	0·155	0·093	1·67
	0·142	0·071	2·00	*	*	*
Mean	**0·131**	**0·073**	**1·79**	**0·182**	**0·111**	**1·64**
Mean q_{Ac}		**0·204**			**0·293**	

* Sample lost.

is known to inhibit the inactivation of DPN by nucleotidase (Mann & Quastel, 1941; Handler & Klein, 1942), and this property was confirmed in rat mammary tissue by Terner (1952). Nicotinamide might therefore neutralize the inhibitory effect of glucose on acetate oxidation by conserving endogenous DPN. In experiments with rat mammary slices metabolizing acetate and glucose (Table 4) the presence of nicotinamide increased the rate of oxidation of both carbons of acetate by a factor of 1·5–2, but had no appreciable effect on the q_{CO_2H}/q_{Me} ratio. These results are therefore in agreement with the hypothesis that there is competition for DPN between the systems responsible for glucose catabolism and acetate oxidation, and this competition could be responsible for the fact that glucose depresses the rate of oxidation of acetate total carbon (q_{Ac}) whereas pyruvate does not.

Effect of medium. Popják & Tietz (1954) found that both pyruvate and glucose, added to rat mammary slices metabolizing acetate in phosphate–saline, increased the rate of oxidation of (carboxyl-labelled) acetate by as much as a factor of 7. Because their results were very different from those described in the present paper, one experiment was set up with phosphate–saline. It was found that glucose increased the mean value (four flasks) of q_{CO_2H} from 0·018 to 0·127, i.e. an increase comparable with that reported by Popják & Tietz (1954). This suggests that, in the work with bicarbonate–saline, the reduction in the rates of oxidation of acetate carbon atoms is connected with the presence of the bicarbonate ion. This is consistent with the idea that the dilution of cycle intermediates postulated by Strisower *et al.* (1952) occurs by β-carboxylation of pyruvate.

It will be noted that, when acetate is the only substrate, the rate of oxidation of the carboxyl atom by mammary slices in phosphate–saline is much less than in bicarbonate–saline ($q_{CO_2H} = 0{\cdot}018$ compared with 0·160 as shown in Table 1). In phosphate–saline the effect of glucose is only to increase the value of q_{CO_2H} to about the same value as observed when bicarbonate–saline is used. No further work has yet been done to investigate the reason for these results, but one possibility is that glucose (or pyruvate, as used by Popják & Tietz, 1954) maintains the level of tricarboxylic acid cycle intermediates.

Oxidation of glucose

At the concentration of glucose used in this work the rate of oxidation is known to be independent of concentration (Hirsch *et al.* 1954) and hence the rates of oxidation of acetate and glucose may be directly compared. It is then found that acetate total carbon is oxidized at only about one-fortieth of the rate of glucose total carbon when both substrates are present together (Tables 1 and 5).

Table 5. *Oxidation of total glucose carbon by lactating-rat mammary tissue in presence and absence of acetate*

For details see text.

Expt. no.	Acetate absent q_{Gt}	Acetate present q_{Gt}
36	10·1	8·9
	9·1	9·0
37	7·9	9·6
	8·8	8·9
39	5·2	5·8
	5·6	6·6
40	6·1	5·9
	—	5·6
73	4·3	4·3
	4·4	—
	4·2	4·7
	3·7	4·1
Mean	**6·5**	**6·7**

Table 6. *Oxidation of the* C-1 *atom of glucose by lactating-rat mammary tissue in the presence and absence of acetate*

For details see text.

Expt. no.	Acetate absent $q_{G\text{-}1}$	Acetate present $q_{G\text{-}1}$
56	1·29	1·66
	1·28	1·39
	1·24	1·51
	1·03	—
60	0·72	0·76
	0·62	0·72
	—	0·68
Mean	**1·03**	**1·12**

Table 7. *Oxidation of uniformly labelled glucose by lactating-rat mammary tissue in presence and absence of pyruvate*

For details see text.

Expt. no.	Pyruvate absent q_{Gt}	Pyruvate present q_{Gt}
52	1·47	0·81
	2·21	1·34
	2·45	1·25
Mean	**2·04**	**1·13**
78	5·22	1·76
	6·25	1·88
	6·42	1·55
Mean	**5·97**	**1·73**

It is known, from the relative rates of incorporation of glucose and acetate carbon into fatty acids (Balmain *et al.* 1954), that glucose gives rise to C_2 units about 1·5 times as fast as does acetate. Hence, if glycolysis were the only pathway of glucose catabolism in lactating-rat mammary tissue one would expect the $C_{(1)}$ atom of glucose to be oxidized at a similar rate to that of the methyl carbon of acetate. Experiment shows, however, that the $C_{(1)}$ atom is oxidized about 10 times as fast as the methyl carbon (Tables 6 and 1). This indicates that most of the $C_{(1)}$ atoms of the glucose molecules which appear as carbon dioxide must be oxidized other than by way of C_2 units.

It also seems unlikely that β-carboxylation at the pyruvate stage of the glycolytic pathway can account for an appreciable part of the glucose $C_{(1)}$ atoms oxidized. Calculations based on the theory of Strisower *et al.* (1952) show that when glucose is added to slices metabolizing acetate the influx of carbon into the cycle from sources other than acetate increased by an amount corresponding to only 17% of the acetate oxidized. And the rate of oxidation of the methyl carbon of acetate is already only about one-tenth of that of the glucose $C_{(1)}$ atom to which it corresponds. Even if the theory of Strisower *et al.* (1952) were not wholly valid (and certain discrepancies have been noted in the present work) it is clearly impossible for β-carboxylation to account for a large proportion of the $C_{(1)}$ atoms oxidized without the resultant dilution causing a much greater reduction in the observed rate of oxidation of labelled acetate.

These observations are consistent, at least qualitatively, with the observations of Abraham *et al.* (1954), who calculate that the direct oxidative pathway (Dickens, 1953) accounts for about 60% of the total glucose catabolized in lactating-rat mammary tissue. Independent evidence has been provided by Glock & McLean (1954), who find that the levels of the enzymes of this pathway in lactating-rat mammary tissue begin to rise rapidly immediately after parturition and reach a peak between the 10th and 15th day of lactation, i.e. at the stage of lactation at which tissue for this work was taken. The level of these enzymes is then much higher than in any other of the many tissues examined.

If, as thus seems virtually certain, the direct oxidative pathway is active in lactating-rat mammary tissue the effect of pyruvate on the rate of oxidation of glucose is anomalous. It would be expected to produce a reduction in the rate of oxidation of glucose total carbon because of dilution at the pyruvate stage of the glycolytic pathway, and, as shown in Table 7, this is what is found. But it would not be expected to cause any reduction in the rate of oxidation of the $C_{(1)}$ atoms, whose oxidation must occur, for reasons stated above, mainly by a route not involving pyruvate. Experiment shows, however, that pyruvate reduces the value of $q_{G\text{-}1}$ to less than half (Table 8). This is difficult to explain. It does not seem likely to be due to competition for a coenzyme. The reactions of the direct oxidative pathway leading to the appearance of the $C_{(1)}$ atom as carbon dioxide require triphosphopyridine nucleotide, a coenzyme not involved in the catabolism of pyruvate. Hirsch *et al.* (1954) have expressed the view that pyruvate has an inhibitory effect on glucose oxidation apart from any effect due to isotopic dilution but, if that is so, then the inhibition must operate on both catabolic pathways, or on some earlier common stage. One possibility is that added pyruvate undergoes synthetic reactions and dilutes the glucose carbon, possibly at the glucose 6-phosphate stage, before it is catabolized by either pathway. These possibilities are being investigated.

Table 8. *Oxidation of the* C-1 *atom of glucose by lactating-rat mammary tissue in the presence and absence of pyruvate*

For details see text and Table 6.

Expt. no.	Pyruvate absent $q_{G\text{-}1}$	Pyruvate present $q_{G\text{-}1}$
61	1·01	0·45
	1·08	0·41
	1·13	0·40
	1·21	0·39
78	1·09	0·50
	1·03	0·41
	1·31	0·48
Mean	**1·12**	**0·44**

SUMMARY

1. By the use of doubly labelled sodium acetate ($^{14}CH_3.^{13}CO_2Na$) and of glucose labelled uniformly and in the C-1 atom the rates of oxidation of acetate and glucose carbon by lactating-rat mammary slices have been measured in the presence and absence of other substances.

2. The ratio of the rates of oxidation of the acetate carbon atoms (q_{CO_2H}/q_{Me}) is increased by the addition of either glucose or pyruvate, but whereas glucose also depresses the total rate of oxidation of acetate pyruvate does not.

3. The formula of Strisower *et al.* (1952), which accounts for a change in the ratio of the rates of oxidation of the acetate carbon atoms in terms of dilution of isotope in the tricarboxylic acid cycle,

has been applied to these results, and certain discrepancies have been observed. It is suggested that these might be due to competition for diphosphopyridine nucleotide and this possibility has been tested by the use of added diphosphopyridine nucleotide and nicotinamide.

4. From a comparison of the relative rates of oxidation of the C-1 atom of glucose and the methyl atom of acetate it is concluded that most of the $C_{(1)}$ atoms must be catabolized by a route other than glycolysis, this route probably being the direct oxidative pathway.

5. If the direct oxidative pathway is therefore quantitatively important in this tissue the depressing effect of pyruvate on the rate of oxidation of the $C_{(1)}$ atom of glucose is anomalous. Possible reasons for the effect are discussed.

Our thanks are due to Dr J. R. Catch of the Radiochemical Centre, Amersham, for the gift of a sample of [1-^{14}C]glucose used in preliminary experiments and received before it was generally available, to Dr D. H. Tomlin for carrying out the ^{13}C determinations and to Mr B. W. E. Peaple for technical assistance.

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Studies on the Metabolism of Mammary Tissue *in vitro*

2. OXIDATION OF ACETATE AND GLUCOSE BY SLICES OF LACTATING SHEEP MAMMARY GLAND

By W. G. DUNCOMBE and R. F. GLASCOCK
National Institute for Research in Dairying, University of Reading

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Earlier work in which the manometric technique was used has shown that ruminant mammary tissue metabolizing acetate as the only substrate has a respiratory quotient (R.Q.) above unity (about 1·2), which is increased (to about 1·4) by the addition of glucose (Folley & French, 1950). In a medium containing only glucose as substrate, however, the R.Q. was found to be less than unity. These results were interpreted as meaning that the tissue is virtually inert to carbohydrate, whereas acetate can supply both the carbon and the energy for fatty acid synthesis. The possibility was recognized, however, that in the presence of both substrates glucose carbon might also be used. Later work in which isotopes were used showed that lactating-sheep mammary tissue actively incorporates the carboxyl carbon of acetate into fatty acids when it is the only substrate, and even more actively when glucose is also present (Balmain, Folley & Glascock, 1952). Further (Balmain, Folley & Glascock, 1954), it was found that sheep mammary slices use about 30 times as much acetate carbon as glucose carbon for fatty acid synthesis when both substrates are present in the medium.

As noted in Part 1 of this series (Duncombe & Glascock, 1956), the manometric technique does not indicate the relative amounts of each substrate oxidized when more than one is present, and in the later work only isotope incorporation into fatty acids was measured. Similar experiments to those carried out on lactating-rat mammary tissue have therefore been carried out on sheep tissue. Some of this work has already been communicated in preliminary form (Duncombe & Glascock, 1953), and the delay in accumulating sufficient results for this paper has been due chiefly to the fact that lactating-sheep tissue can be obtained only in the spring.

EXPERIMENTAL

Animals. All the ewes were in their first lactation and each with a single lamb. They were killed by shooting through the brain on the 21st to 28th day of lactation.

Substrates. As before, the labelled substrates used were doubly labelled sodium acetate ($^{14}CH_3.^{13}CO_2Na$) of specific activity 40 μmc/mg. of methyl C and ^{13}C abundance 68 atom % excess in carboxyl carbon; uniformly ^{14}C-labelled and 1-^{14}C-labelled glucose of specific activity 8·5 μmc/mg. Unlabelled sodium acetate, sodium pyruvate and glucose were also used. The concentrations used in the media were: glucose 300 mg./100 ml. (0·0167 M); sodium acetate and sodium pyruvate, 0·02 M. The specific activity of the CO_2 obtained by combustion of the substrates was determined with the same gas counter as was used to measure the specific activity of the CO_2 produced in the experiments. This was of the order of 200 counts/min./ml. (about 0·15 μmc/ml.) of CO_2 in the experiments with labelled glucose and 1000 counts/min./ml. (about 0·7 μmc/ml.) of CO_2 in the experiments with labelled acetate. In the latter the ^{13}C abundance in the CO_2 was from 1 to 4 atoms % excess.

Incubation. The udder tissue was sliced with a hand microtome and batches of slices (0·5 g.) were incubated for 3 hr. at 37° with shaking in bicarbonate–saline (Krebs & Henseleit, 1932), in which the necessary substrates had been dissolved; the gas phase was O_2+CO_2 (95:5). The procedure was exactly as described for rat mammary tissue (Duncombe & Glascock, 1956). Briefly, it consists in the use of an incubation unit fitted with stopcocks and standard joints. At the end of the incubation the unit is attached to a high-vacuum apparatus, acid is introduced through a side arm and the liberated CO_2 separated, measured, and the abundance of isotopes in it determined. ^{14}C was determined with a gas counter and ^{13}C determined in the mass spectrometer. The whole procedure has been described in detail by Glascock (1954).

RESULTS

The rate of substrate oxidation is reported here as μg. of carbon/mg. dry wt. of tissue/hr., and is designated in the tables by the symbol q with the appropriate subscripts. Thus q_{CO_2H} and q_{Me} refer to the rates of oxidation of the carboxyl and methyl-carbon atoms of acetate; q_{Ac} refers to the rate of oxidation of acetate total carbon (so that $q_{Ac}=q_{CO_2H}+q_{Me}$); q_{Gt} refers to the rate of oxidation of glucose total carbon and $q_{G\text{-}1}$ to that of the $C_{(1)}$ atom of glucose. In the tables, L and R refer to left and right udder halves of the same animal.

Oxidation of glucose

Although the absolute level of oxidation of glucose total carbon is quite variable between animals, there is, in nearly all experiments, a well-marked depression when pyruvate is added (Table 1). By contrast the oxidation of the $C_{(1)}$ atom of glucose is unaffected (Table 2).

The oxidation of both glucose total carbon and the C-1 atom is increased several times on the addition of acetate (Tables 3 and 4). The apparently low metabolic activity of animal 80 is attributed to the abnormally high fat content of the tissue.

Table 1. *Effect of pyruvate on the oxidation of glucose total carbon by lactating-sheep mammary slices*

Expt. no.	Pyruvate absent q_{Gt}	Pyruvate present q_{Gt}
54	1·64	0·89
	2·19	0·91
	2·06	0·90
Mean	**1·96**	**0·90**
80 L	0·103	0·048
	0·103	*
80 R	0·118	0·042
	0·065	0·093
Mean	**0·109**	**0·061**

* Sample lost.

Table 2. *Effect of pyruvate on the oxidation of the glucose $C_{(1)}$ atom by lactating-sheep mammary slices*

Expt. no.	Pyruvate absent $q_{G\text{-}1}$	Pyruvate present $q_{G\text{-}1}$
54	0·19	0·21
	0·21	0·21
	0·20	0·18
Mean	**0·20**	**0·20**
80 L	0·015	0·017
	0·014	0·017
80 R	0·034	0·043
	0·017	0·028
Mean	**0·020**	**0·025**

Table 3. *Effect of acetate on the oxidation of glucose total carbon by lactating-sheep mammary slices*

Expt. no.	Acetate absent q_{Gt}	Acetate present q_{Gt}
30 L	1·89	3·33
30 R	1·39	2·94
31	1·51	3·24
32 L	1·08	3·44
32 R	1·11	2·60
33 L	2·31	11·20
33 R	1·24	4·83
Mean	**1·50**	**4·51**

Table 4. *Effect of acetate on the oxidation of the glucose $C_{(1)}$ atom by lactating-sheep mammary slices*

Expt. no.	Acetate absent $q_{G\text{-}1}$	Acetate present $q_{G\text{-}1}$
53	0·22	0·93
	0·20	1·08
	0·23	0·84
	0·24	0·93
Mean	**0·223**	**0·945**
80 L	0·015	0·029
	0·014	0·046
80 R	—	0·028
	0·017	0·023
Mean	**0·0153**	**0·0315**

Oxidation of acetate

As will be seen from Table 5, the addition of glucose to the incubation medium considerably increased the rates of oxidation of both carbon atoms of acetate. These were affected about equally, so that although q_{Ac} rose from 0·783 to 1·145 the ratio q_{CO_2H}/q_{Me} was unaltered.

In the work on rat tissue (Duncombe & Glascock, 1956) the formula of Strisower, Kohler & Chaikoff (1952) was used to calculate the degree of dilution of isotopic tricarboxylic acid cycle intermediates which occurs by an influx of unlabelled molecules during the metabolism of labelled acetate. This is given by

$$x = \frac{(q_{CO_2H}/q_{Me} - 1)}{(q_{CO_2H}/q_{Me} + 1)} \times 100,$$

where x, the degree of dilution, is defined by the statement that after dilution x% of the molecules of cycle intermediates have been derived from unlabelled molecules. On applying this equation to the results in Table 5, it is found that x has values of 19·0 and 17·0 in the absence and presence of added glucose. Thus, whereas in rat tissue the effect of glucose was to depress the rate of oxidation of acetate and to increase the degree of dilution (x) in sheep tissue there is an increase in the rate of acetate oxidation and virtually no change in the degree of dilution.

Effect of pyruvate. Pyruvate has an effect quite different from that of glucose (Table 6). The rate of oxidation of both carbon atoms of acetate is depressed so that q_{Ac} is reduced from 0·783 to

Table 5. *Effect of glucose on acetate oxidation by lactating-sheep mammary slices*

	Glucose absent			Glucose present		
Expt. no.	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$
30 L	2·17	—	—	2·45	—	—
30 R	0·61	—	—	1·56	—	—
31	0·89	—	—	1·34	—	—
32 L	1·34	—	—	2·22	—	—
32 R	1·17	—	—	1·56	—	—
33 R	1·44	—	—	2·78	—	—
57	0·540	0·390	1·38	0·590	0·450	1·31
	0·560	0·400	1·40	0·950	0·690	1·38
	0·420	0·290	1·45	0·520	*	—
	0·500	0·340	1·47	0·730	0·550	1·33
75 L	0·278	0·180	1·54	0·611	0·426	1·44
	0·284	0·175	1·62	0·364	0·233	1·56
75 R	0·629	0·414	1·52	0·883	0·599	1·47
	0·514	0·344	1·49	0·628	0·452	1·39
Mean†	**0·466**	**0·317**	**1·48**	**0·659**	**0·486**	**1·41**
Mean† q_{Ac}		**0·783**			**1·145**	
x		**19·0**			**17·0**	

* Sample lost.

† Mean Expts. 57, 75 L and 75 R.

Table 6. *Effect of pyruvate on acetate oxidation by lactating-sheep mammary slices*

	Pyruvate absent			Pyruvate present		
Expt. no.	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$	q_{CO_2H}	q_{Me}	$\frac{q_{CO_2H}}{q_{Me}}$
57	0·540	0·390	1·38	0·490	0·270	1·81
	0·560	0·400	1·40	0·410	0·230	1·78
	0·420	0·290	1·45	0·480	0·280	1·71
	0·500	0·340	1·47	*	—	—
75 L	0·278	0·180	1·54	0·362	0·190	1·91
	0·284	0·175	1·62	0·269	0·146	1·84
75 R	0·629	0·414	1·52	0·258	0·142	1·82
	0·514	0·344	1·49	0·368	0·168	2·19
Mean	**0·466**	**0·317**	**1·48**	**0·377**	**0·204**	**1·86**
Mean q_{Ac}		**0·783**			**0·581**	
x		**19·0**			**30·1**	

* Sample lost.

0·581. At the same time the ratio q_{CO_2H}/q_{Me} is increased from 1·48 to 1·86, the corresponding values of x being 19·0 and 30·1.

DISCUSSION

The appreciable rate of oxidation of glucose by sheep mammary tissue found here is in contrast to the findings of Folley & French (1950), whose respiratory experiments indicated that this tissue is almost inert to glucose in the absence of other substrates. It is found further that the addition of pyruvate considerably depresses the rate of oxidation of glucose total carbon while having no effect on the rate of oxidation of the $C_{(1)}$ atom. On this evidence alone it might be concluded that glucose is catabolized by the direct oxidative pathway (Dickens, 1953) in which the conversion of the $C_{(1)}$ atom into carbon dioxide does not involve pyruvate. However, consideration of the relative rates of oxidation of glucose total carbon and the $C_{(1)}$ atom place serious difficulties in the way of accepting this mechanism. For each molecule of carbon dioxide derived from the $C_{(1)}$ atom of glucose by the direct oxidative pathway there is left a 5-carbon residue. It is highly improbable that this would be completely oxidized, but, if it were, the ratio of the rate of oxidation of glucose total carbon (q_{Gt}) to that of the $C_{(1)}$ atom ($q_{G\text{-}1}$) could not be more than 6:1. If any of the 5-carbon residue escaped oxidation, this ratio would be smaller. The results show, however (Tables 1 and 2), that the ratio is as much as 10:1 (Expt. 54). Thus a major part of the glucose oxidized cannot be catabolized by way of the direct oxidative pathway. In support of this is the fact (Glock & McLean, 1954 and private communication) that the levels of enzymes of this pathway are much lower in sheep than in rat mammary tissue.

It thus seems likely that glycolysis may be the major pathway of glucose catabolism in sheep mammary tissue, and indeed Dr C. Terner (private communication) has found evidence for it from manometric experiments. However, several inconsistencies remain to be resolved: the effect of added pyruvate reported here and the fact that glucose is a poor source of C_2 units in this tissue (Balmain *et al.* 1954) would not be expected if glycolysis were the major catabolic pathway. Furthermore, the great increase in the rate of oxidation of glucose on the addition of acetate cannot be accounted for on the present evidence.

The effects of glucose and of pyruvate on acetate oxidation are also inconsistent. If the theory of Strisower *et al.* (1952) is tenable, then the fact that the degree of dilution x (see Results) is not increased on the addition of glucose means that glucose does not give rise to tricarboxylic acid cycle intermediates. Pyruvate, on the other hand, does increase the value of x, implying that it can give rise to such intermediates. This suggests a block between glucose and pyruvate which, again, would not be expected if glycolysis were the major catabolic pathway.

Species differences. The present results, together with those of Part 1 (summarized for convenience in Table 7), give further evidence of the considerable metabolic differences between mammary tissue of rats and sheep. In agreement with earlier work on acetate utilization (e.g. Folley & French, 1949, 1950), it is now found that the rate of acetate oxidation is several times as fast in sheep as in rat mammary tissue; at the same time the effects on acetate oxidation of added pyruvate or glucose are

Table 7. *Summary of observed species differences between lactating rat and sheep mammary slices in the oxidation of acetate and glucose, and in the interaction of substrates*

	Effect on acetate metabolism	
Additions	Rat tissue	Sheep tissue
Acetate	$q_{Ac}=0{\cdot}273$	$q_{Ac}=0{\cdot}783$
Acetate + glucose	q_{Ac} reduced by 50%; ratio* increased	q_{Ac} increased by 50%; ratio unaffected
Acetate + pyruvate	q_{Ac} unaffected; ratio increased from 1·54 to 1·93	q_{Ac} reduced by 25%; ratio increased from 1·48 to 1·86
	Effect on glucose metabolism	
Additions	Rat tissue	Sheep tissue
Glucose	$q_{Gt}=6{\cdot}5$ $q_{G\text{-}1}=1{\cdot}03$	†$q_{Gt}=1{\cdot}96$ †$q_{G\text{-}1}=0{\cdot}2$
Glucose + acetate	No effect	q_{Gt} and $q_{G\text{-}1}$ increased threefold
Glucose + pyruvate	q_{Gt} and $q_{G\text{-}1}$ reduced by a factor of 2–3	q_{Gt} reduced by 50%; $q_{G\text{-}1}$ not affected

* Ratio = ratio of rates of oxidation of acetate carbon atoms q_{CO_2H}/q_{Me}.
† Taken from Tables 1 and 2 respectively (results for Expt. 54).

qualitatively different in the two species. By contrast, sheep tissue oxidizes considerably less glucose than does rat tissue, though in the presence of acetate the levels are comparable; as already mentioned, this level of glucose oxidation was not suspected from the results of earlier manometric experiments. The effects on glucose oxidation of added acetate or pyruvate are also very different in the two species. It is now clear, however, that glucose must serve as an important source of energy in the lactating mammary gland of the sheep especially when, as in the living animal, acetate is present.

These marked species differences are of particular interest in view of the conclusions drawn by Hirsch, Baruch & Chaikoff (1954) from experiments on lactating-rat mammary gland which were confirmed in this laboratory (see Duncombe & Glascock, 1956). They say: 'The increase in glucose oxidation that results from the addition of glucose to the medium shifts the metabolism of the C_2 intermediate, derived from acetate and pyruvate, from an oxidative fate to one involving synthesis.'

It is now clear that this statement does not apply even approximately to what is observed in sheep tissue under identical experimental conditions. As already noted, the addition of glucose causes an increase in acetate oxidation (Table 5), and also in the rate of synthesis of fatty acids from acetate (Balmain *et al.* 1952). Thus there is no question of a shift in the metabolic fate of acetate; glucose increases its utilization for both synthesis and oxidation.

It may therefore be that the statement of Hirsch *et al.* (1954) is only a brief summary of their observations and is not a precise description of what is believed to occur in the tissue. This seems all the more likely since, as shown by their own work, the decrease in the rate of acetate oxidation consequent on the addition of glucose is, at the most, only about one-tenth of that needed to compensate for the increased rate of fatty acid synthesis.

SUMMARY

1. The rates of oxidation of the two carbon atoms of sodium acetate, glucose total carbon and the $C_{(1)}$ atom of glucose have been measured in lactating-sheep mammary slices in the presence and absence of other substrates.

2. The rates of oxidation of the two acetate carbon atoms are increased by about 50% on addition of glucose to the medium, the ratio of their rates of oxidation being unaffected. Pyruvate, on the other hand, causes a reduction in the rate of acetate oxidation and an increase in the ratio of the rates of oxidation of the two carbon atoms (q_{CO_2H}/q_{Me}).

3. The formula of Strisower *et al.* (1952) applied to these results indicates that whereas pyruvate can give rise to tricarboxylic acid cycle intermediates, glucose cannot do so and suggests that there is a metabolic block between glucose and pyruvate in this tissue.

4. Studies on the oxidation of glucose in the presence and absence of pyruvate, however, do not confirm this conclusion, neither do they indicate that the direct oxidative pathway is a major route of glucose catabolism.

5. The effect of acetate on glucose oxidation has also been investigated, and is found to increase it threefold, glucose and acetate thus increasing the rate of oxidation of each other.

6. The metabolic behaviour of sheep mammary tissue now observed is compared with that of rat mammary tissue previously reported.

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Some Properties of the Actomyosin-like Protein of the Uterus

By D. M. NEEDHAM and J. M. CAWKWELL
Department of Biochemistry, University of Cambridge

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Comparatively little work has been done on the protein extractable from uterus muscle under conditions similar to those used for extracting actomyosin from skeletal muscle. Csapo (1949, 1950*a*, *b*) has used 0·5 M-KCl extracts of rat, rabbit and human uterus and, by comparing the viscosity before and after addition of adenosine triphosphate (ATP) with the viscosity of skeletal-muscle extracts similarly treated, has concluded that uterus muscle contains actomyosin but less than that in skeletal muscle. Csapo (1949, 1950*a*, *b*) has correlated this actomyosin content with functional activity in different states of the uterus. Schwalm, Cretius & Lange (1955) have studied variations in the viscosity response to ATP of extracts made with M-KCl from human uteri in different states.

With extracts considered, from their similar specific viscosity after ATP addition, to contain similar amounts of myosin, Csapo (1949) found that the adenosine triphosphatase (ATPase) activity was less in those from the uterus than from skeletal muscle. He also made purified preparations by dilution precipitation of the protein soluble in 0·5 M-KCl; the ATPase of such preparations, like skeletal-muscle actomyosin, was activated more by Ca^{2+} than by Mg^{2+} ions. In all this work ATPase activity was measured by a viscosimetric method, and from the results only approximate values can be deduced for the enzymic activity in terms of inorganic P liberated/mg. of actomyosin N. Westgren (1950) has also prepared protein from human, cow and rabbit myometrium, following the method of Szent-Györgyi (1951) for preparation of myosin B. He found the ATPase of the once-precipitated material to be activated by either Mg^{2+} or Ca^{2+} ions, rather more by the latter; he gives a pH–activity curve with several peaks, the most important being about 7·3 and 8·5, and suggests that more than one ATPase might be present. Since no protein concentrations are reported, it is not possible to compare the ATPase activity with the values of other workers.

In connexion with work which we are doing on the proteins of the uterus, it seemed desirable to make a more detailed and quantitative study of the ATPase activity of uterus actomyosin. Csapo and Westgren seem to have assumed that the protein they were dealing with from smooth muscle was the same as the actomyosin of striated muscle. Crepax (1952), on the other hand, studying the electrophoretic behaviour of 0·5 M-KCl extracts of uterus muscle, considers the proteins present to be different. Snellman & Tenow (1954) also believe the contractile element of the uterus to have a different protein composition from that of skeletal muscle. In the present paper the term actomyosin will be used, for convenience, for the protein prepared from the uterus by the methods ordinarily used for the preparation of myosin B from skeletal muscle; this is done without any intention of deciding at the present stage the question of the identity of the uterus protein.

Singher & Millman (1947), using rat uterus, reported that homogenates of the whole organ showed three times as great ATPase activity as homogenates of skeletal muscle. In the present work it has been found that only a small part of this activity can be due to actomyosin; the greater part is due to cell particles which can be centrifuged down at 40000 to 50000 *g*. In such homogenates the particles are derived from the endometrium as well as the myometrium and, as will be shown below, the ATPase activity of cell particles from rat uterus is much higher than that of particles from pig or rabbit myometrium. Nevertheless, in the smooth-muscle cells of the myometrium the particulate fraction (8–9 % of the total N) is likely to be quantitatively more important than in many skeletal muscles—certainly than in rabbit skeletal muscle ordinarily used for actomyosin preparation (Chappell & Perry, 1953). In preparing uterus actomyosin we have therefore taken care to remove the particulate fraction from the extract before precipitation of the actomyosin by dilution. A study of the characteristics of actomyosin and of cell-particle ATPase from the whole uterus or the myometrium alone shows that the two enzymes can be clearly distinguished.

A few experiments were made on the viscosity of the actomyosin before and after ATP addition, to make certain that our preparations gave the expected changes.

A preliminary report of this work has been published (Needham & Cawkwell, 1955).

EXPERIMENTAL

Materials

Homogenate of rat uterus for the study of ATPase distribution. This was made by grinding rat uteri (all selected at the stage of late oestrus) with 10 vol. of 1·25 M-KCl, containing 0·08 % ATP (sodium salt) and buffered with 0·03 M-$NaHCO_3$ at pH 7·9. In order to facilitate the disintegration, the cooled tissue was allowed slowly to freeze solid in air at −15°, and the frozen material was ground with the medium and about 0·5 g. of acid-washed sand/g. All subsequent work was done at about 4°. After measured samples had been removed, the homogenate was centrifuged at 600 *g*, and extracted twice more, each time with 5 vol. The combined supernatants were centrifuged at 600 *g* to remove any remaining debris, and then at 41 000 *g* for 1 hr. to sediment the cell particles. All values given for *g* are average values. Uniform suspensions in 0·5 M-KCl were made of the particle pellet and of the residue.

Preparation of actomyosin. This was made by a method based on those described by Bailey (1942), Szent-Györgyi (1951), Portzehl, Schramm & Weber (1950) and Hasselbach & Schneider (1950–51) for the preparation of myosin B. Pregnant uterus was used in all cases; pig material for preparation I was taken after 40 days' pregnancy; for preparations II and III after 30 days. The rabbit and rat material was taken very near term. Glass-distilled water was used for all solutions and for the precipitation by dilution.

The sections of pig uterus to be used were cooled and brought to the laboratory as quickly as possible. All further work was carried out in a cold room at 4°. After being slit open, the uterus was scraped free from endometrium and the myometrium was peeled away as much as possible from the outer fibrous layer. Histological examination showed that the material used was nearly all myometrium. This was minced and a weighed amount of about 50 g. was homogenized in a Waring Blendor with 3 vol. of 0·5 M-KCl containing 0·08 % ATP (sodium salt) and 0·05 M-KH_2PO_4; the latter had been brought to pH 7·5 by addition of N-KOH. The homogenization was for six periods of 30 sec., with 1 min. intervals. This stage was reached about 1·5 hr. after the death of the animal.

The homogenate was stirred slowly for some hours, then left at 0° overnight. Next day the extremely viscous suspension was further diluted with 1 vol. of extractant and then centrifuged at 3000 *g*. The supernatant was filtered with gentle suction through a layer of paper pulp (1–2 cm.) previously saturated with extractant. The filtrate was centrifuged in the Spinco ultracentrifuge, model L. Table 1 shows the effect of filtering and of duration and speed of centrifuging on the protein-N content of the dilution precipitates and of the particle material spun down. In later work, in order to hasten the preparation, the filtration was omitted and centrifuging in the Spinco centrifuge for 1 hr. at 105 000 *g* was used as a routine.

The supernatant was first diluted about fivefold with water at 0° and the pH was adjusted, by means of 0·5 N acetic acid, to 6·5–6·7, with the glass electrode. The dilution was then continued to 14-fold. A precipitate appeared which quickly flocculated. The preparation was allowed to stand at 0° overnight, then the clear fluid was decanted and the suspension centrifuged. The precipitate was dissolved in about 40 ml. of 0·6 M-KCl, any traces of undissolved matter being spun off. The precipitation by dilution to 0·04 M-KCl was repeated once or twice more, care being taken that the pH remained about 6·6. Finally, the preparation was stored at 0° as a solution in 0·5 M-KCl containing about 1 mg. of actomyosin N/ml. The pH was brought to 7·0 by means of a few drops of 0·6 M glyoxaline buffer.

With the uteri from rabbit and rat the initial stages could be carried out more quickly. With the rat the whole uterus was used after washing and blotting.

The cell-particle suspension. This was made by adding a few drops of cold 0·5 M-KCl to the pellet in the centrifuge tube and working up gently with a glass rod to a uniform suspension; more solution was gradually added up to the required volume.

Special chemicals. ATP was used as the sodium salt supplied by the Sigma Chemical Co.

Glyoxaline (from L. Light and Co., Ltd.) was sometimes coloured faintly yellow. On these occasions it was recrystallized first from benzene, then from ethanol.

2:4-Dinitrophenol (DNP) was recrystallized first from ethanol, then from water–ethanol (50 %, v/v).

$MgCl_2$ and $CaCl_2$ solutions were made from A.R. reagents and standardized by titration with silver nitrate.

Table 1. *Effect of various treatments on the amount of cell-particle preparation and actomyosin precipitate*

Pig preparation I was used. Filtration was through paper pulp as described in the text; the unfiltered extract had a preliminary centrifuging at 600 *g* for 20 min. to remove cell debris. Centrifuging in the Spinco centrifuge was for 1 hr. at 41 000 *g*; or for 1 hr. at 41 000 *g*, followed by 1 hr. at 105 000 *g*.

	Period of centrifuging (hr.)	Protein N derived from 1 g. wet wt. of myometrium (mg.)
Whole extract, unfiltered	—	10·3
Whole extract, filtered	—	9·7
Actomyosin pptd. from filtered extract	—	1·30
Sediment from unfiltered extract	1	0·83
	2	1·72
Sediment from filtered extract	1	0·40
	2	1·00
Actomyosin pptd. from filtered, centrifuged extract	1	1·48
	2	0·88

Methods of estimation

Inorganic P was estimated by the method of Fiske & Subbarow (1925) or, for quantities below 20 μg., by the method of Weil-Malherbe & Green (1951). Protein N was estimated by the micro-Kjeldahl method of Chibnall, Rees & Williams (1943).

Ribonucleic acid (RNA) was estimated by a modification of the method of Schmidt & Thannhauser (1945). Phospholipids were first removed by the method of P. D. Mitchell and J. M. Moyle (personal communication); the mixture was extracted three times with an equal volume of ice-cold *n*-butanol, being allowed to stand each time in the centrifuge tube at 0° for 20 min. with frequent stirring; the layers were

separated by centrifuging, and after removal of the last butanol layer the aqueous phase was treated with cold trichloroacetic acid to a concentration of 5% to remove acid-soluble P; the precipitate was washed twice with 5% trichloroacetic acid, the temperature being kept about 0°. After separation of the deoxyribo- and ribo-nucleic acids, the ribonucleic acid P was estimated by Allen's (1940) method for total P, or if the amounts were below 20 μg., by a modification of the method of Weil-Malherbe & Green (1951). In this case, incineration was done in the presence of 0·3 ml. of conc. H_2SO_4 and 0·2 ml. of 6N perchloric acid (Hanes & Isherwood, 1949); any pyrophosphate formed was hydrolysed by diluting to about 3 ml. and heating for some minutes at 100°. No further acid was added, ammonium molybdate solution in water taking the place of the usual acid molybdate; shaking with *iso*butanol for 5 min. was necessary for complete extraction of the phosphomolybdate under these conditions.

Measurements of enzyme activity

Measurement of ATPase activity. The reaction mixture (final volume 2 ml.) contained unless otherwise stated 0·002M ATP; 0·066M glyoxaline buffer, pH 7·0; and 0·01M-$CaCl_2$. KCl was always present and the concentration is given for the different experiments. The reaction was started by addition of 0·2 ml. containing 0·07–0·2 mg. of actomyosin N, or 0·1 mg. or less of particle N. At these concentrations the rate of reaction was found to be proportional to the amount of enzyme present. The reaction time was 5 min. and the temperature 28°. It was ascertained that the rate remained constant during this period. The reaction was stopped by addition of 1 ml. of trichloroacetic acid (15%, w/v).

Testing of lecithinase activity. Purified α-toxin (from *Clostridium welchii*) dissolved in equal parts (v/v) of 1% NaCl and glycerol was obtained from the Wellcome Research Laboratories through the kindness of Mrs Irene Batty. The solution contained 100 Lf units/ml. (van Heyningen, 1948) or, very approximately, 1·7 Keilley–Meyerhof units (Keilley & Meyerhof, 1950). The preparation was shown to be highly active by the turbidimetric method of van Heyningen (1941), with 1·6 Lf units, 0·8 ml. of egg-yolk solution, 0·04M glyoxaline buffer and 0·001M-$CaCl_2$ in 1·6 ml. At 25° maximum turbidity appeared in 15 min. Addition of particle suspension (about 0·18 mg. of N) had no effect on the development of turbidity.

Viscosity measurements

An Ostwald viscosimeter was used, about 8 ml. in capacity, with a flow-time of 88 sec. for water at 18°.

RESULTS

ATPase activity. For the preliminary experiments with homogenates of rat uterus the results given in Table 2 are typical. They show that about 60% of the ATPase activity is due to the particulate material. Moreover, most of the activity of the residue is probably due also to cell particles not

Table 2. *Distribution of ATPase activity in rat-uterus homogenate*

The preparation of the fractions and the reaction mixture was as described in the text. The KCl concn. was adjusted to 0·1M. Incubation was for 3 min. at 25°. The measured samples of homogenate or tissue fraction added to the reaction medium contained 0·08–0·13 mg. of N.

	P liberated/g. wet wt. of uterus (mg.)
Homogenate	2·83
Particulate fraction	1·72
Supernatant	0·42
Residue	0·84

Table 3. *Effect of* KCl *concentration on ATPase activity of actomyosin and cell-particle preparations from the uterus*

The conditions of incubation and the composition of the reaction mixture were as described in the text.

		Inorganic P liberated/mg. of N/min. (μmoles)	
		With 0·1M-KCl	With 0·6M-KCl
Pig I	Actomyosin, once pptd.*	0·38	0·68
Pig II	Actomyosin, once pptd.*	0·24	0·48
	Actomyosin, twice pptd.*	0·28	0·56
	Particle preparation	0·56	0·49
Pig III	Actomyosin, twice pptd.	0·34	0·63
	Actomyosin, twice pptd.†	0·094	0·49
	Particle preparation†	0·60	0·48
Rat	Actomyosin, twice pptd.	0·56	0·52
	Particle preparation	7·5	5·4
Rabbit	Actomyosin, three-times pptd.	0·19	0·70
	Particle preparation	1·10	1·10

* Actomyosin pptd. from extracts before high-speed centrifuging. In the other cases centrifuging was for 1 hr. at 105000 *g*.

† 0·001M-$CaCl_2$ used.

removed in the extraction; even after seven extractions it was found that the residue still contained about 20% of the total ribonucleic acid. The ATPase activity of the supernatant containing the actomyosin was only about 15% of the whole.

The ATPase activity of all the uterus-actomyosin preparations tested (Tables 3 and 4) is very low compared with the activity of actomyosin from skeletal muscle under similar conditions. Thus Greville & Needham (1955) give values of about 6 μmoles of P liberated/mg. of N/min. at 25° in 0·1 M-KCl, pH 7·0. The ATPase activity of the cell-particle preparations was higher, especially in the rat; these very high values have been found in many experiments with rat preparations. Raising the KCl concentration to 0·4 M or above about doubles the ATPase activity of the actomyosin preparations (with the exception of the rat preparation) whilst having only a slight inhibitory effect on the cell-particle preparations. With the uterus actomyosin from the rat, several KCl concentrations between 0·025 M and 0·6 M were tried without affecting the ATPase activity.

Another clear distinction between the actomyosin and the particulate ATPase is to be found in the effect of Ca^{2+} and Mg^{2+} ions. In every case Ca^{2+} ions activate actomyosin ATPase much more strongly than Mg^{2+} ions, whilst sometimes the one ion, sometimes the other, is slightly the better activator of cell particles (Table 5).

2:4-Dinitrophenol ($3–5 \times 10^{-3}$ M) also, in the presence of 0·6 M-KCl, raises the actomyosin-ATPase rate considerably, without much affecting the particle ATPase (Table 6). The behaviour of the rat material is again anomalous in that actomyosin activation is also obtained at low KCl concentrations, and this will be discussed later.

The effect of pH upon the activity in 0·6 M-KCl of uterus actomyosin from the rabbit is shown in Fig. 1. Three buffers, 0·06 M citrate, 0·035 M glyoxaline and 0·05 M aminotrishydroxymethylmethane (tris) were used, overlapping to cover the pH

Table 4. *Effect of* KCl *concentration on the ATPase activity of uterus actomyosin*

The composition of the reaction mixture was as described in the text. The actomyosin (pig II) was precipitated twice from extract which had been centrifuged for 1 hr. at 105000 *g*.

Concn. of KCl (M)	Inorganic P liberated/mg. of N/min. (μmole)
0·1	0·16
0·2	0·17
0·4	0·50
0·6	0·51
0·8	0·50
1·0	0·48

Table 5. *Effect of* Ca^{2+} *and* Mg^{2+} *ions on ATPase activity of actomyosin and particle preparations from the uterus*

The conditions of incubation and the composition of the reaction mixture were as described in the text. The actomyosin preparations were all made from extracts which had been centrifuged for 1 hr. at 105000 *g*; they were precipitated twice, except for the rabbit preparation, which was precipitated three times.

			Inorganic P liberated/mg. of N/min. (μmoles)	
		Activator	With 0·1 M-KCl	With 0·6 M-KCl
Pig II	Actomyosin	0·01 M-$CaCl_2$	0·16	0·58
		0·002 M-$MgCl_2$	0·10	0·09
	Particle prep.	0·01 M-$CaCl_2$	0·56	0·49
		0·002 M-$MgCl_2$	0·68	0·63
Pig III	Actomyosin	0·001 M-$CaCl_2$	—	0·67
		0·01 M-$CaCl_2$	—	0·76
		0·001 M-$MgCl_2$	—	0·069
		0·01 M-$MgCl_2$	—	0·069
	Particle prep.	0·001 M-$CaCl_2$	0·30	0·25
		0·001 M-$MgCl_2$	0·30	0·30
Rat	Actomyosin	0·01 M-$CaCl_2$	0·46	—
		0·002 M-$MgCl_2$	0·037	—
	Particle prep.	0·01 M-$CaCl_2$	7·5	—
		0·002 M-$MgCl_2$	5·6	—
Rabbit	Actomyosin	0·01 M-$CaCl_2$	0·19	—
		0·001 M-$MgCl_2$	0·019	—
	Particle prep.	0·01 M-$CaCl_2$	1·10	1·10
		0·001 M-$MgCl_2$	1·55	1·30

Table 6. *Effect of 2:4-dinitrophenol on ATPase activity of actomyosin and particle preparations from the uterus*

The conditions of incubation and the composition of the reaction mixture in the control were the same as described in the text. The actomyosin and particle preparations were the same as those used in Table 5.

		Concn. of DNP (mM)	Inorganic P liberated/mg. of N/min. (μmoles) With 0·1 M-KCl	With 0·6 M-KCl
Pig II	Actomyosin	—	—	0·36
		3·5	—	0·52
	Particle prep.	—	—	0·56
		3·5	—	0·53
Pig III	Actomyosin	—	0·34	0·76
		3·5	0·28	1·22
		—*	—	0·069
		3·5*	—	0·12
	Particle prep.	—	0·54	—
		3·5	0·54	—
		4	0·24	—
Rat	Actomyosin	—	0·51	0·38
		3·5	0·87	0·87
	Particle prep.	—	—	0·54
		3·5	—	0·54
Rabbit	Actomyosin	—	0·19	0·63
		3	0·20	1·47
		5	—	1·88
	Particle prep.	—	—	0·74
		3	—	0·90
		5	—	1·01

* 0·001 M-$MgCl_2$ was used as activator.

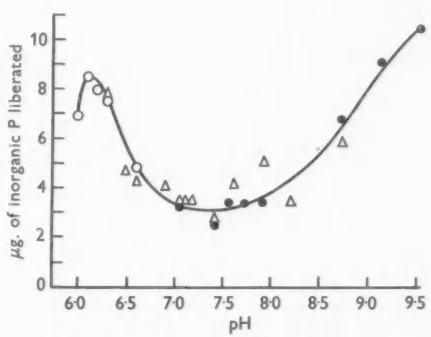

Fig. 1. pH–Activity curve for uterus actomyosin. The preparation was from the rabbit and had been three times precipitated; 0·1 mg. of actomyosin N was used, in a final volume of 2 ml. The reaction medium contained 0·002 M ATP (sodium salt), 0·01 M-$CaCl_2$ and 0·6 M-KCl. The buffers used were 0·05 M tris (●); 0·035 M glyoxaline (△); 0·06 M citrate (○). Reaction time, 3 min. at 25°.

Table 7. *Ribonucleic acid content of actomyosin and particle preparations from the uterus*

The actomyosin and particle preparations were the same as those used in Table 5. The ATPase activities used are those obtained in the usual conditions (see text) with 0·6 M-KCl.

		RNA P/mg. of N (μg.)	Inorganic P liberated/μg. of RNA P/min. (μmole)
Pig II	Actomyosin	11·1	0·047
	Particle prep.	51·0	0·011
Pig III	Actomyosin	11·5	0·055
	Particle prep.	60·0	0·010

range 6·0–9·5. There was usually good agreement between the rates with different buffers at any given pH.

In some experiments the ribonucleic acid content of the particle preparations was estimated and compared with that of the actomyosin preparations. It is not certain that all the RNA content of

the actomyosin was due to contamination by particles, but a maximum value for possible contamination is obtained. Calculation of the ATPase activity/μg. of RNA P in each case showed that the activity of the actomyosin preparations was 4–5 times that which could be due to particulate matter carried down (Table 7).

Myokinase. Myokinase was found to be absent from the actomyosin tested, pig preparation III (Fig. 2). The liberation of inorganic P ceased after half the easily hydrolysable P had been split off, in spite of the addition of more enzyme at 30 min. With the cell-particle preparation, on the other hand (also from pig III and washed once with 0·5 M-KCl), the liberation of inorganic P continued steadily well beyond the half-way point. This indicates either that myokinase was present as well as ATPase, or that the particle preparation contained an apyrase capable of splitting off two phosphate groups from ATP.

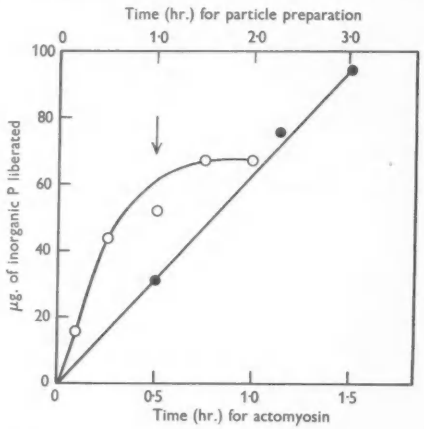

Fig. 2. Testing of actomyosin and washed-particle preparation for myokinase. The medium (final volume 2 ml.) contained 0·001 M ATP (sodium salt); 0·01 M-$CaCl_2$; 0·6 M-KCl and 0·066 M glyoxaline buffer. At the arrow a second addition of actomyosin solution was made. Temp. 28°. ○, Actomyosin; ●, particle preparation.

Effect of lecithinase. In the early stages of this work, before the activating effect of high KCl concentration and of DNP on the actomyosin ATPase was known, it was thought that it might be possible to confirm the activity of actomyosin ATPase from the rat, as distinct from the very active particle ATPase, by the susceptibility of the latter to lecithinase (Keilley & Meyerhof, 1948, 1950). It was, however, not found possible to cause more than 10–20 % inhibition of the ATPase of the particle preparation, although the conditions, described in the Experimental section as optimum for egg-yolk flocculation, were varied by increasing the lecithinase content, by increasing the time of action to 1 hr. and by substituting histidine buffer (as used in the experiments of Keilley & Meyerhof) for the glyoxaline buffer. Thus a typical particle preparation (0·8 mg. of N) lost about 20 % of its ATPase activity on incubation for 1 hr. with 4 Lf units of lecithinase in 1·6 ml. final volume; the control, similarly treated but without lecithinase, lost 9 %. The percentage effect on ATPase activity was the same whether Ca^{2+} or Mg^{2+} ions were used as activator in testing the ATPase.

Viscosity changes. Pig preparation I was tested at three stages for viscosity changes on ATP addition, and the results are shown in Table 8. The values for specific viscosity and for the fall caused by ATP are somewhat greater than those recorded by Snellman & Tenow (1954) for pregnant uterus, but considerably smaller than those given by Csapo (1949). The value for Z (see Table 8) can

Table 8. *Changes in viscosity of uterus actomyosin on ATP addition*

The actomyosin was from pig I, and was dissolved in 0·6 M-KCl containing 0·05 M-KH_2PO_4 brought to pH 7·0 with N-KOH. Soln. A contained actomyosin pptd. from filtered extract before high-speed centrifuging. Soln. B contained actomyosin pptd. from the extract after centrifuging for 1 hr. at 41000 *g*. Soln. C contained actomyosin pptd. from extract after centrifuging for 1 hr. at 41000 *g*, followed by 1 hr. at 105000 *g*. A portion (8 ml.) of solution was used in each case, the outflow time for the buffer being 85 sec. The volume of 0·1 M ATP added was 0·1 ml. Temp. 18°.

	Specific viscosity			Protein concn. (%)	Z_η* before ATP	ATP sensitivity (%)*
	Before ATP	After ATP	Returning to			
Soln. A	0·67	0·38	0·67	0·45	0·11	57
Soln. B	0·74	0·38	0·74	0·45	0·13	64
Soln. C	0·37	0·24	0·37	0·26	0·078	40

* $Z_\eta = \dfrac{2{\cdot}3 \times \log \eta_{rel.}}{\text{concn. (g./l.)}}$. ATP sensitivity (%) $= \dfrac{\log \eta_{rel.} - \log \eta_{rel.\ ATP}}{\log \eta_{rel.\ ATP}} \times 100$,

where $\eta_{rel.}$ and $\eta_{rel.\ ATP}$ are the relative viscosities before and after addition of ATP (Portzehl *et al.* 1950).

be compared with that of about 0·3 given by Portzehl *et al.* (1950) for actomyosin from skeletal muscle under similar conditions; these authors give values up to 165 for the percentage ATP sensitivity of actomyosin from skeletal muscle.

DISCUSSION

Evidence has been presented that the ATPase activity of actomyosin preparations from the uterus is due to the actomyosin itself and not to contaminant particles, and also that a specific ATPase is concerned.

The behaviour of the uterus-actomyosin ATPase shows some resemblances to that of striated-muscle actomyosin. The viscosity changes on ATP addition are of course similar, though on a smaller scale. If we assume that the uterus actomyosin in 0·6 M-KCl is readily dissociated into actin and a myosin-like protein, then this myosin-like protein, in the same way as the myosin of skeletal muscle, is activated by DNP (Greville & Needham, 1955; Chappell & Perry, 1955), and its pH–activity curve is very similar (Mommaerts & Green, 1954). There are, however, some differences in behaviour; thus, skeletal-muscle actomyosin is known to show, under the conditions similar to those used in this work, decreasing ATPase activity with increasing KCl concentration (Mommaerts & Green, 1954; Greville & Needham, 1955). This is taken to mean that at pH 7·0 the ATPase activity of the undissociated actomyosin is greater than that of myosin. With uterus actomyosin the increased activity with high KCl concentrations would indicate that the myosin-like protein has greater ATPase activity than the actomyosin. Uterus actomyosin from the rat is anomalous here, being neither activated nor much inhibited by high KCl concentrations, and being activated by DNP at low KCl concentrations as well as at high. This might indicate that this actomyosin is dissociated with particular ease.

In this work we found that actomyosin ATPase keeps its activity for at least 2–3 weeks. The loss of actomyosin shown in Table 1 which occurred on prolonged high-speed centrifuging was also noticed with actomyosin from skeletal muscle. Crepax (1952) comments on the instability of the uterus proteins soluble in solutions of high ionic strength and contrasts this behaviour with that of the proteins from skeletal muscle; but it is not made clear wherein the instability consists.

The amount of protein obtained as actomyosin from the myometrium by the method described was very small, only 1·0–1·5 mg. of N/g. wet wt. after two precipitations. No quantitative figures seem available for the yield from skeletal muscle after comparable treatment. This yield from the myometrium was certainly not quantitative. Experiments with whole uterus from the pregnant rat by Robinson's (1952) method for complete extraction and precipitation with ethanol (15%, v/v) gave values of 3·5–4·5 mg. of actomyosin N/g. wet wt.; these are to be compared with a content of about 18 mg. of actomyosin N/g. wet wt. for skeletal muscle (Hasselbach & Schneider, 1950–51).

The cell particles after the treatment of the tissue as described for actomyosin extraction are no doubt in a damaged condition, and it is to be expected that their ATPase activity would be fully unmasked (cf. Keilley & Keilley, 1951). The lack of activation by DNP is in agreement with this. With mitochondria from pigeon-breast muscle showing no latent ATPase activity, Chappell & Perry (1953) found consistently greater activation by Mg^{2+} ions than by Ca^{2+} ions, especially at concentrations of 0·01 M and below. With the cell particles from the uterus as used in this work, the superiority of Mg^{2+} ion activation was neither so consistent nor so great.

The ATPase activity of the particles from rat uterus is exceptionally high. Chappell & Perry (1953) found about 1·5 μmoles of P liberated/mg. of N/min. for unmasked mitochondria from pigeon-breast muscle; and Lardy & Wellman (1953) found 2·5 μmoles for mitochondria from rat liver in the presence of DNP. The resistance of the particulate ATPase to lecithinase attack was unexpected, since the material from rat muscle found by Keilley & Meyerhof (1948) to contain the lecithinase-sensitive enzyme was centrifuged down between 10000 and 18000 *g* and was considered by them to be microsomal in origin; these particles, like those in our work, must have been damaged by their previous treatment. The different behaviour of the uterus particles might depend on the difference in tissue or on differences in previous treatment. This matter is being investigated further.

SUMMARY

1. An actomyosin-like protein prepared from pig and rabbit myometrium and from whole uterus of the rat showed specific adenosine-triphosphatase activity. In 0·1 M-KCl at pH 7·0 this activity was 3–10% of that of skeletal-muscle actomyosin under similar conditions. Like the latter, it was activated by Ca^{2+} ions much more than by Mg^{2+} ions. A pH–activity curve is given.

2. With the protein from pig and rabbit myometrium, the activity was about twice as high in 0·4–0·8 M-KCl as in 0·1 M-KCl. This behaviour contrasts with that of skeletal-muscle actomyosin, which is inhibited by raising the KCl concentration. In the presence of high KCl concentration there was activation by $3–5 \times 10^{-3}$ M 2:4-dinitrophenol, as with skeletal-muscle actomyosin.

3. The actomyosin-like protein from rat uterus showed some anomalies, being neither activated nor inhibited by changing the KCl concentration, and being activated by 2:4-dinitrophenol at low as well as high KCl concentrations.

4. In the course of the actomyosin preparation, a cell-particle fraction was separated; its adenosinetriphosphatase activity showed very different characteristics from those of the actomyosin.

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Metabolism of Calcium, Phosphorus and Nitrogen in Hypervitaminosis A in Young Rats

By M. K. NERURKAR and M. B. SAHASRABUDHE
Biology Division, Department of Atomic Energy, Government of India, Indian Cancer Research Centre, Parel, Bombay 12, *India*

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The production of toxic effects in human beings as well as in experimental animals by the administration of large doses of pure vitamin A is well recognized clinically (Josephs, 1944; Knudson & Rothman, 1953). Among the experimental animals, rats appear to be specially sensitive to these toxic effects. Skeletal fractures and haemorrhages are among the important features in this malady (Herbst, Pevcek & Elvehjem, 1944; Moore & Wang, 1945; Pevcek, Herbst & Elvehjem, 1945). Although the skeletal changes that lead to these fractures have been known for some time, the mechanism remains obscure. Further, since previous workers' preparations of vitamin A contained large amounts of vitamin D, it is not known with certainty whether the recorded effects are due to vitamin A or to vitamin D. Histological studies by Wolbach (1947) suggested that accelerated epiphyseal cartilage sequences and remodelling processes are responsible for the production of fractures, and that these are mediated independently of the pituitary (Wolbach & Maddock, 1952). Fell & Mellanby (1950), in their investigations of tissue cultures of bone, have shown that vitamin A acts directly. In an attempt to elucidate the mechanism of action of pure vitamin A, free from vitamin D, on bones, the effects of hypervitaminosis A on the chemical composition of bones

and on the general metabolism of calcium, phosphorus and nitrogen in rats have been studied. The results are reported in the present paper.

MATERIALS AND METHODS

The experimental animals were male, albino inbred rats, 6–7 weeks' old and fed on a synthetic diet having the following percentage composition: sugar, 34·5; starch, 34·5; casein, 20·0; vegetable fat, 6·7; salt mixture (Hawk & Oser, 1931), 4·0; cystine, 0·2; choline hydrochloride, 0·1. The diet was further supplemented with 10 units of vitamin D, 100 units of vitamin A, 1 mg. of vitamin E, 0·05 mg. of vitamin K and adequate amounts of B complex. Analysis of the diet showed 0·692 % Ca, 0·440 % P and 2·430 % nitrogen.

Synthetic vitamin A palmitate (1 million i.u./g. Hoffmann–La Roche) was used as the source of vitamin A in all these experiments. As this source of vitamin A is free from vitamin D, the possibility of introducing the condition of hypervitaminosis D (Moore & Wang, 1943, 1945) is ruled out in these experiments. Vitamin A (40000 i.u./rat/day) was given to these animals orally.

Metabolic studies were carried out on male rats, kept in pairs in metabolism cages provided with a device for separate collection of urine and faeces. Urine was collected into 10 ml. of a preservative consisting of equal amounts of toluene and conc. HCl. Urine collected during 2 days was pooled for assay purposes. At the end of each collection period, the cages and funnels were washed with hot water acidified with HCl to pH 4·6–4·8 and the washings pooled with the urine. Faeces were collected separately. Samples of urine and faeces were stored at 7–8° until analysed for calcium, total phosphorus and total nitrogen. These experiments were divided into three parts: (1) a preliminary control period of 10 days; (2) a period of administration of excess of vitamin A (10 days); (3) a curative period of 14 days after the administration of excess of vitamin A was stopped. The animals were weighed on alternate days. Food intake was measured daily. An amount somewhat above the daily expected intake was weighed out and put in the cages. At the end of 24 hr., the intake was calculated after weighing the residue. Food waste and its contamination with excreta were reduced by using a special feeding device on the cages. The animals had free access to water.

In another type of experiment, five groups of male albino rats 45–50 days old were used. One group served as the control, whereas the other groups received 40000 i.u. of vitamin A every day, for 3, 6, 9 and 12 days respectively. X-ray photographs of the whole body were taken at intervals. The animals were killed 24 hr. after receiving the last dose, by decapitation, without previous fasting. The chest wall was opened and blood withdrawn from the heart into a heparinized syringe. The levels of calcium, inorganic phosphorus and vitamin A in the serum were investigated. The livers were removed, weighed and placed in 5% ethanolic KOH solution for the estimation of vitamin A content. The femora and tibiae were cut out and cleaned of all the adhering soft tissues. Slits were made longitudinally through the diaphysis and epiphysis to render the extraction more effective. The bones were defatted with two overnight extractions, first with absolute ethanol and then with ether. The dried marrow was removed as completely as possible. The bones, free of fat and marrow, were then dried in an electric oven at 105°, powdered and analysed for Ca, P and N according to the procedure outlined below.

Analytical methods

Nitrogen. N was estimated by the micro-Kjeldahl method as modified by Sobel, Yuska & Cohen (1937).

Calcium. Faeces, food and bones were ashed (A.O.A.C. 1940) before the determinations. The following methods were employed: for faeces and food, the method of Lindner & Kirk (1937): for urine, the same method after a previous treatment with potassium persulphate (Shohl & Pedley, 1922); for bone, titration directly with disodium dihydrogen ethylenediaminetetraacetate with murexide indicator. The procedure was developed by us and its principle is the same as that for the estimation of blood Ca described by Holtz & Seekles (1952). This latter method was used for the estimations in blood.

Phosphorus. Total P in urine, faeces, food or bones was estimated by the method of Fiske & Subbarow (1925), as modified by LePage & Umbreit (1949).

Faeces, food and bones were ashed as for the Ca determinations. Inorganic P in the blood serum was determined by the method of Fiske & Subbarow (1925).

Vitamin A was estimated in liver by the method of Gallup & Hoefer (1946) by saponification and extraction of vitamin A with light petroleum. Vitamin A in blood was estimated according to the technique of Yudkin (1941). Characteristic absorption was measured by means of a Beckman quartz spectrophotometer (Zscheile & Henry, 1942) with Morton & Stubbs' (1948) correction. Vitamin A (U.S.P.) was used as a reference standard (U.S. Pharmacopoeia, 1948).

RESULTS

Administration of single toxic doses of vitamin A resulted in general drowsiness and muscular weakness, accompanied by decreased activity of the animals. If no further excess of vitamin A was given they returned to normal physical condition within 24 hr. Continued administration of vitamin A at toxic dosage for a period of several days caused symptoms such as lack of appetite, loss of weight, muscular weakness, loss of hair, soreness and bleeding of the eyes, stiffness of the limbs, limping, spontaneous fractures, internal haemorrhages and eventually death. Death occurred in a high proportion of animals given more than

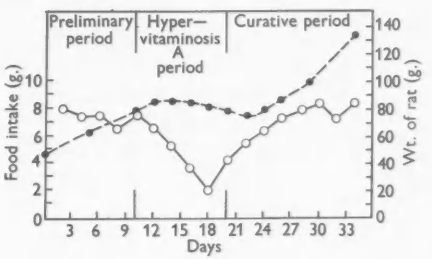

Fig. 1. Effect of hypervitaminosis A on food intake (○) and body weight (●) of rats. Dose given: 40 000 i.u. of vitamin A/day/rat.

Table 1. *Effect of hypervitaminosis A on the excretion of calcium, phosphorus and nitrogen in urine and faeces*

The dose of vitamin A was 40000 i.u./rat/day during the hypervitaminosis A period. The figures are the averages of results from eight rats, Each figure corresponds to a period of 2 days.

	Calcium				Phosphorus				Nitrogen			
	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)	Intake (mg.)	Urinary excretion (mg.)	Faecal excretion (mg.)	Net retention (% intake)
Preliminary period (10 days)*	110·0	7·2	31·3	65·0	70·0	20·6	7·1	60·4	386	61·6	14·5	80·3
	101·0	6·5	27·6	66·4	64·6	20·6	6·3	58·4	357	51·9	17·1	80·7
	102·0	5·5	26·8	68·4	65·1	22·0	5·4	57·7	359	54·8	16·3	80·2
	90·0	5·2	26·9	64·3	57·2	18·7	4·4	59·6	315	53·1	16·8	77·8
	103·0	6·1	28·5	66·4	65·5	21·0	6·4	58·1	362	60·3	20·5	77·6
Period of hypervitaminosis A (10 days)†	90·9	7·1	34·9	53·2	57·1	22·5	6·3	49·5	306	66·8	18·7	72·1
	72·0	8·2	39·3	33·7	45·7	23·0	6·7	35·3	252	89·5	15·8	58·2
	50·5	8·9	39·3	4·37	31·8	22·0	8·0	5·68	177	114·0	14·4	27·7
	25·8	7·2	25·9	−21·0	18·8	22·8	12·5	−82·2	99	118·0	18·0	−51·7
	57·3	15·8	46·0	−7·91	36·5	25·8	12·3	−4·7	201	165·0	20·7	+8·0
Curative period (14 days)‡	75·6	14·5	56·9	5·58	47·8	28·3	10·1	19·6	266	220·0	23·5	8·76
	87·0	14·2	54·7	20·5	57·4	30·5	10·8	27·9	306	167·0	25·2	36·9
	101·0	15·5	65·4	19·8	64·2	27·7	7·6	44·6	355	185·0	21·6	41·7
	110·0	13·7	48·9	43·1	70·6	23·2	9·3	53·9	391	171·0	22·0	50·7
	115·0	11·5	55·3	42·2	73·5	21·7	9·5	58·2	405	147·0	19·8	59·0
	101·0	10·7	41·6	48·2	64·2	18·7	7·6	59·0	354	128·0	18·4	61·4
	118·0	12·7	43·8	52·6	75·3	21·2	11·0	57·2	415	98·0	17·8	72·1

* Preliminary period when animals were maintained on adequate diet.
† Period when the animals were given 40000 i.u. of vitamin A/day in addition to adequate diet.
‡ Period when excess of vitamin A was withdrawn.

twelve doses of vitamin A. In some animals, abnormal posture was observed, with marked bending of the spine, both hind legs appearing paralysed but without any apparent signs of fractures or haemorrhages. The results regarding food intake and growth rate of animals are shown in Fig. 1.

Results of metabolic studies on the urinary and faecal excretions of calcium, phosphorus and nitrogen are given in Table 1. It will be seen that there is a gradual decrease in the percentage retentions of all the components with the continuation of excess of vitamin A administration. There is a negative balance of all the three constituents in the second half of the vitamin A administration period which suggests increased catabolism. The rapidity and the extent of the negative balance closely paralleled the physical condition of the animals. It will be seen from Table 1 that the levels of calcium, phosphorus and nitrogen excretions follow a more or less identical pattern.

Table 2 shows the results of the effect of hypervitaminosis A on the calcium, phosphorus, nitrogen and ash content of the bones. The figures are expressed as percentages of water-free, fat-free and marrow-free bones. The first group of rats, which had received three doses of vitamin A on 3 consecutive days and which were killed on the fourth day were very little different from the control group. The differences become apparent in the second group, which received excess of vitamin A for 6 consecutive days. The maximum effects are seen in the group dosed for 9 days, the group dosed for 12 days showing slightly less difference from the controls than this group. Systematic X-ray examination of the animals at various stages of the experiment showed gradual thinning of the bones. Although bone calcium and phosphorus

Table 2. *Effects of hypervitaminosis A on the composition of bones*

The dose was 40000 i.u. of vitamin A/rat/day. The analyses for calcium, phosphorus and nitrogen are given on dry, fat-free and marrow-free bone.

Treatment of group	No. of animals	Ca (%)	P (%)	N (%)	Ash (%)
Control	12	26·1±0·145	12·4±0·095	4·02±0·027	69·1±0·176
3 doses	6	25·5±0·121	11·8±0·115	4·18±0·045	69·0±0·150
6 doses	6	25·1±0·170	11·5±0·156	4·23±0·030	68·4±0·218
9 doses	6	23·7±0·176	10·3±0·167	5·09±0·051	66·0±0·375
12 doses	6	24·5±0·215	11·0±0·210	4·86±0·042	67·5±0·272

Table 3. *Levels of calcium and inorganic phosphorus in blood serum of normal rats and rats with hypervitaminosis*

The dose was 40000 i.u. of vitamin A/rat/day.

		Serum Ca		Inorganic P	
Treatment of group	No. of animals	Average value (mg./100 ml.)	Range (mg./100 ml.)	Average value (mg./100 ml.)	Range (mg./100 ml.)
Control	12	10·5±0·215	9·21–11·75	4·54±0·093	3·98–5·01
3 doses	6	10·8±0·394	9·65–11·97	4·61±0·197	4·10–5·16
6 doses	6	10·6±0·483	8·81–11·93	4·73±0·169	4·15–5·25
9 doses	6	11·2±0·381	9·92–12·13	4·86±0·206	4·19–5·45
12 doses	6	10·9±0·324	9·85–11·98	5·05±0·178	4·43–5·65

Table 4. *Levels of total vitamin A in the blood and liver of normal rats and rats with hypervitaminosis*

The dose of vitamin A was at the rate of 40000 i.u./day for each animal.

Treatment of group	No. of animals	Total vitamin A received (i.u.)	Blood vitamin A (i.u./ml.)	Liver vitamin A (i.u./g.)	Liver vitamin A (Total i.u.)	Storage in liver (%)*
Control	12	—	66·10±2·84	75±3·02	418±10·8	—
3 doses	6	120 000	184·0±8·36	2750±88·4	13 300±630·0	11·12
6 doses	6	240 000	211·0±7·14	3870±97·0	22 300±930·0	9·30
9 doses	6	360 000	237·0±6·85	5000±120·0	27 300±907·0	7·57
12 doses	6	480 000	262·0±4·92	6000±71·5	31 400±990·0	6·54
15 doses	6	600 000	308·0±5·50	6810±39·5	35 200±445·0	5·86

* Percentage of the administered dose of vitamin A found in liver.

gradually decrease, the levels of calcium and inorganic phosphorus in the serum remain unaltered, as can be seen from Table 3.

Table 4 shows the concentration of total vitamin A (vitamin A alcohol and ester) in the liver and blood after various doses of the vitamin. The purpose of these estimations was to ascertain indirectly whether the absorption of vitamin A is impaired as a result of the massive dosage. Since liver is the chief storage site of vitamin A (Osborne & Mendel, 1918; McCoord & Clausen, 1934) the concentration of vitamin in this organ should be proportional to the intake (Baumann, Riising & Steenbock, 1934) and should serve as a criterion of its absorption (Guggenheim & Koch, 1944; Foy & Morgareidge, 1948). It will be observed from these results that the levels of vitamin A in the blood increased with increasing period of treatment with vitamin A, but no direct correlation could be demonstrated between total amount of vitamin A administered and that present in either the blood or the liver. Although the vitamin A content of the liver in experimental rats increased with the number of doses of vitamin A administered, the absorption, calculated as percentage of the total dose administered, decreased, indicating decreased absorption of vitamin A from the intestine, or increased catabolism of liver vitamin A, or an approach to the saturation limit of the liver. The vitamin A content of the liver in the 15-dose group is greater than that in the previous group (12 doses); thus the liver was not completely saturated.

DISCUSSION

The results described above indicate that pure vitamin A free from vitamin D is toxic when given orally at a dose of 40000 i.u./day. The toxic effects observed were similar to those observed by other workers (Moore & Wang, 1945; Pevcek *et al.*1945). Reduced food intake, loss of weight, skeletal fractures and haemorrhages are in our experience the most characteristic features in genuine hypervitaminosis A. Fractures occur most consistently in young growing rats and were found without exception in rats fed for 12 or more days with 40000 i.u. of vitamin A/day. Vitamin A in the concentrations employed in these investigations appears to interfere in some way with the metabolism of calcium, phosphorus and nitrogen. The retention of these elements in the body is decreased and there is a negative balance. The severity of the interference is more or less parallel with the amount of vitamin A ingested, since the degree of negative balance of calcium, phosphorus and nitrogen becomes more and more pronounced during continued ingestion of the vitamin. This may be due to decreased absorption from the digestive tract or to an increased catabolic excretion from the system or to both; but it is not possible to decide from measurements of the excretion of these elements in urine and in faeces whether absorption from the digestive tract is interfered with or whether there is increased catabolic excretion (Brown & Shohl, 1930). This is particularly true for calcium, because systemic calcium is excreted in urine, by way of bile, etc., and in the faeces. The fraction in the faeces which comes from the catabolism of systemic calcium is known as 'endogenous faecal calcium' to distinguish it from 'exogenous faecal calcium', which is the unabsorbed calcium from the food (Schmidt & Greenberg, 1935). Because of this inherent difficulty in assessing the absorption of calcium from the intestines, we cannot say whether the calcium absorption is impaired or not. But there is no doubt that administration of large doses of vitamin A induces a negative balance in calcium, phosphorus and nitrogen. Recovery from the deleterious effects is evidently slow. The balance has not returned to normal even 10 days after the cessation of administration of excess of vitamin A.

In the early stages of hypervitaminosis A there is a definite rise in the excretion of both urinary and faecal calcium, phosphorus and nitrogen. But in the later stages, although the doses are continued, there appears, contrary to our expectations, to be a fall in the urinary and faecal excretions of all these elements. The reason for this decrease, which coincides with the decrease in the levels of calcium in bones, cannot be ascertained.

The process of bone formation consists of several phases, one of which is the precipitation of calcium phosphate. For this to occur, there must be adequate concentrations of calcium and inorganic phosphorus in the blood stream and alkaline phosphatase in the bones (Howard, 1951). The fact that in our experiments the serum concentrations of calcium and phosphorus are not disturbed and that the bone alkaline-phosphatase activity is increased in hypervitaminosis A (Nerurkar & Sahasrabudhe, unpublished work) suggests that this phase of ossification is not impaired and that the development of fragility and fractures must be ascribed to the disturbances in other phases of bone formation. Our observations that no marked changes in the relative chemical composition of the bones are apparent as a result of hypervitaminosis A are in agreement with those of Moore & Wang (1945) and Rodahl (1950). Gradual thinning of the bones, observed by us in X-ray photographs, has also been reported by Wolbach (1947) and Metre (1947). This gradual thinning of the bones, coupled with the pronounced negative balance, suggests that hypervitaminosis A is draining the inorganic stores of the bones without causing any gross change in their relative composition.

SUMMARY

1. Oral administration of a pure vitamin A preparation at a dose 400 times the normal requirement has been shown to produce effects such as reduced food intake, loss of weight, skeletal fractures and haemorrhages in young albino rats. The degree of toxicity is approximately proportional to the total quantity of vitamin A administered.

2. The effects of administration of large doses of vitamin A on the metabolism of calcium, phosphorus and nitrogen have also been studied. A negative balance for calcium, phosphorus and nitrogen sets in and continues for a considerable period after the administration of large doses of vitamin A has ceased.

3. No changes were detected in the levels of calcium and inorganic phosphorus of the blood.

4. Very little change in the relative mineral composition of the bones was observed. Increased excretion of calcium and phosphorus is therefore probably due to thinning of the bones.

5. Estimation of vitamin A in the liver suggests that administration of amounts of vitamin A, several times greater than the normal requirements, reduces the absorption of vitamin A from the intestine.

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An Improved Method for Measuring Degree of Polarization

By W. F. HARRINGTON,* P. JOHNSON and R. H. OTTEWILL
Department of Colloid Science, University of Cambridge

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Measurements of the depolarization of fluorescence have recently been used for determining the rotational relaxation times of protein molecules (Weber, 1952*a*, *b*), and they provide a technique of considerable flexibility and usefulness. Such work requires a high degree of accuracy in the measurement of percentage polarization, which is not readily achieved by the methods hitherto described (Perrin, 1929; Lewis, Lipkin & Magel, 1941; Lewis & Bigeleisen, 1943). In connexion with a recent study (Harrington, Johnson & Ottewill, 1956), an improved apparatus has been devised, based upon a method first proposed by Lyot (1929) and later improved by Wright (1934). Its advantages depend upon the principle of matching the intensity and position of a fringe system in two halves of a divided field, rather than judging the

* Fellow of the National Foundation for Infantile Paralysis 1953–54. Present address: Department of Chemistry, Iowa State College, Ames, Iowa, U.S.A.

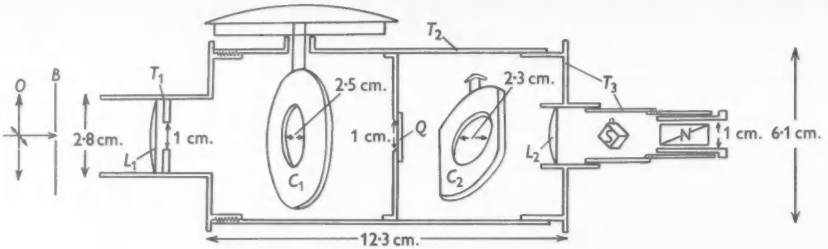

Fig. 1. Diagrammatic representation of polariscope.

complete disappearance of the fringes. Subjectivity and eye strain have thus been drastically reduced, yet the apparatus remains simple and much less expensive than alternative electronic methods (Wille, 1949).

APPARATUS

The apparatus was constructed of three tubular, optically blacked, brass sections, T_1, T_2 and T_3, with dimensions as given in Fig. 1. The plano-convex collimating lens L_1 was of focal length 5 cm. and was fixed with respect to T_2. It was followed immediately by a stop 1 cm. in diameter. The centre section T_2 contained the two compensators C_1 and C_2, mounted on thin annular brass rings, with respective internal diameters of 2·5 and 2·3 cm. Microscope coverslips of Chance glass were cemented to both surfaces of C_1, which could be rotated about a vertical axis (in the plane of the diagram) by means of a Muirhead slow-motion dial graduated from 0° to 270°. A vernier allowed angular positions to be read to 0·1°. Only one coverslip was mounted on C_2, whose axis of rotation, provided with a 'click-stop' at the zero position, was at 45° to that of C_1. The biplate Q was constructed from two quartz sections, one laevorotary and the other dextrorotary, cut normal to the optic axis and cemented side by side to give a divided photometric field, in which the straight junction line was fine and sharp. It was mounted between C_1 and C_2, with its junction line perpendicular to the axis of C_2 and in a plane perpendicular to the axis of T_2. The thickness of the quartz plates was chosen to be 1·76 mm. in order to give rotations for the Hg green line (λ 5461 Å) of +45° and −45°. Lens L_2, of focal length 3 cm., was mounted in an extension tube T_3. A Savart plate S, of such dimensions (1 cm.²) that it gave an angular separation of its interference fringes of 1–2′ of arc (Wright, 1934), was mounted (at the end of a rocking arm) directly behind L_2, and was followed by a Nicol prism N (of square cross-section, 7 mm. side). The tube T_3 could be adjusted inside T_2, and in turn the Nicol prism could be rotated inside T_3. The three-dimensional disposition of components may be seen in Fig. 2.

In setting the Nicol prism with respect to the Savart plate, a distant plane-polarized source was viewed through T_3, from which L_2 had been removed. The tube T_3 was then rotated about its axis (and if necessary N with respect to S) until fringes were obtained. Finally, N was rotated inside T_3 until the best contrast was achieved. L_2 was then replaced in T_3 and the latter adjusted in T_2 until focused on the nearer surface of the biquartz plate. With vertically

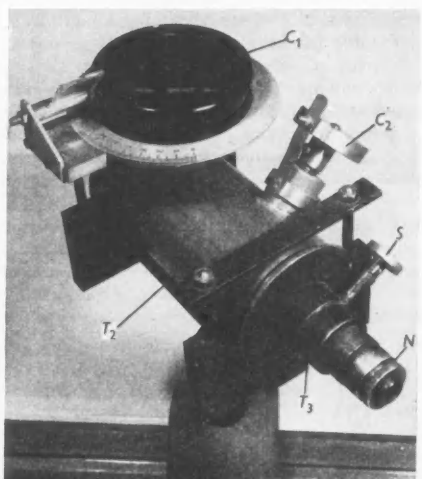

Fig. 2. Photograph illustrating three-dimensional disposition of components.

polarized incident light on stop B and compensators C_1 and C_2 in the zero position, T_3 was then rotated about its axis until the fringes were clear and perpendicular to the junction line of the biquartz.

Optical principles

Assume O is a source of partially plane-polarized light, with the electric vector vertical. By means of stop B and the lens L_1 a parallel beam is obtained through the compensator C_1. The difference in amplitude of the two perpendicular vector components in the beam could therefore be accurately compensated by rotation of C_1 (which preferentially reflects the vertically vibrating component). The angular setting of the compensator necessary to achieve unpolarized light depends on the percentage of plane polarized light in the incident beam and requires calibration (see below).

In the absence of the biquartz Q and compensator C_2 [as in the apparatus of Weber (1952*a*, *b*)] the passage of plane-

polarized light through C_1 is indicated by the observation of typical Savart interference bands, continuous across the whole field. As C_1 is adjusted towards complete compensation (i.e. giving unpolarized transmitted light), the fringes gradually disappear. Such an end point is not in general satisfactory; by introducing the biquartz and compensator C_2 it is replaced by one involving the matching of illuminated halves of the field.

If now C_2 be moved from its zero position (C_1 still giving complete compensation), it imparts a certain small degree of polarization over the whole field, and the fringes in the two halves match in both intensity and position. On the other hand, if C_1 has not given complete compensation, the plane-polarized vector will be rotated $+45°$ in one section of the biquartz and $-45°$ in the other (λ 5461 Å). In passing through C_2, the proportion of polarized component in one half of the field will then be increased, and in the other half decreased, so that the fringes observed through T_3 will no longer match in intensity (because of the difference in degree of polarization) or position (because of the different vibration directions). The compensator C_1 must then be adjusted until matching is complete, and this is facilitated when the fringes are perpendicular to the dividing line of the biquartz. Since the eye is most discriminating when the level of illumination is low, high sensitivity is largely dependent on the introduction of a very small amount of polarization by C_2.

CALIBRATION OF THE APPARATUS

The instrument was calibrated by means of a Watts Standard spectrometer. A 125w mercury arc source was employed, the light of which was diffused by a plate of opal glass, and then made monochromatic by means of an Ilford filter no. 807 (λ 5461 Å). The light through the collimating tube was stopped down at the exit end with a 6 mm. circular stop, giving an unpolarized parallel beam across the spectrometer table. A single glass plate mounted vertically and symmetrically on the spectrometer table was used to obtain transmission polarization. The vertical placing of the glass plate was checked by reflecting the source slit into the spectrometer telescope at various angles. The angle of the plate with respect to the line of the collimator and telescope tube was determined by means of the usual spectrometer techniques. The telescope lenses were then removed and the polarization apparatus was fixed rigidly and carefully as a continuation of the telescope tube. It was then rotated about its own axis until the axis of the first compensator C_1 was perpendicular to that of the glass plate on the spectrometer table. By rotating the spectrometer table through various angles, a variable amount of polarization was introduced into the light beam transmitted through the glass plate. The percentage of plane-polarized light transmitted by the plate was computed from the Fresnel formulae, multiple reflexions being taken into account (Wright, 1934).

The use of undeviated transmitted light from a single glass plate for carrying out the calibration instead of reflected light from the front face of a prism was advantageous, since in the latter case rotation of the polarization apparatus was required. It can be seen from the application of the Fresnel formulae that for a single glass plate a change in polarization for transmitted light of from 0 to 27 % involves an angular change of the plate from 0 to 70°. For reflected light from a prism, the angular change for the same polarization range is from 0 to 10°, and considerable accuracy in angular settings was required.

It was necessary to measure accurately the index of refraction, n_g, of the glass plate in order to compute the percentage polarization by the Fresnel formulae. Although the determination of the refractive index of very thick plates and very thin films is standard procedure, an accurate measurement of n_g for thin plates of approx. 1 mm. is not easily obtained by conventional methods. A method employing a Zeiss interferometer for this measurement was therefore devised.

After calibration of the polariscope, the plate which was used as a source of polarization was cut, fitted into one compartment of the interferometer cell and mounted vertically, with the plane of the plate normal to the light-path. A series of sucrose solutions of varying concentration was prepared and the index of refraction of each determined with an Abbé refractometer to $\pm 0{\cdot}0002$. The fringe displacement was observed for each sucrose solution (both compartments containing the same solution). The equation for the interferometer of the Zeiss type is

$$ln - ln_s = f_0\lambda, \qquad (1)$$

where l is the path length of the double cell, f_0 the number of fringes corresponding to the scale reading for the zero-order band, λ the wavelength of light employed, n the index of refraction of the solution, and n_s that of the solvent.

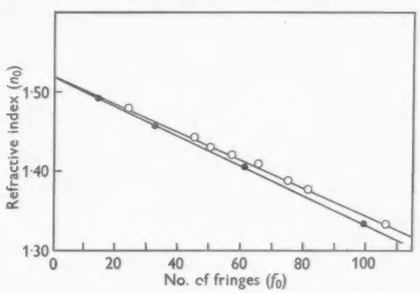

Fig. 3. Determination of refractive index of polarizing glass plates of different thickness.

With the glass plate in position and the same sucrose solution in both compartments of the interferometer, equation (1) may be written

$$(l-d)n_0+n_g d-ln_0=f_0\lambda,$$

or

$$n_0=n_g-f_0\lambda/d, \qquad (2)$$

where n_g is the refractive index of the glass plate, $\frac{1}{2}d$ its thickness and n_0 is the refractive index of the sucrose solution. Thus a plot of n_0 against f_0 gives the refractive index of the plate as the intercept on the n_0 axis (Fig. 3).

RESULTS

In Table 1 is presented a typical set of results, obtained with the polariscope, on a sample of bovine serum albumin which had been coupled with the fluorescent dye 1-dimethylaminonaphthalene-5-sulphonyl chloride. The high degree of reproducibility can be clearly seen, indicating that measurements of the percentage polarization of fluorescence can be made with a standard deviation less than 1% of the percentage polarization. Results with the apparatus have already been published (Harrington *et al.* 1956).

Table 1. *Results obtained on bovine serum albumin conjugate with the polariscope* (pH, 7·3; I, 0·05)

Temp.	Polarization (%)	Mean	S.D.
4·8°	17·4, 17·1, 17·2, 17·2	17·23	0·11
11·3	16·9, 17·0, 16·7, 16·9	16·88	0·11
16·6	16·5, 16·3, 16·2, 16·2	16·30	0·12
22·9	15·5, 15·4, 15·5, 15·6	15·50	0·07
27·7	14·5, 14·7, 14·8, 14·6	14·65	0·11
33·6	14·4, 14·4, 14·5, 14·4	14·43	0·04
40·2	13·3, 13·4, 13·6, 13·5	13·45	0·11

SUMMARY

An improved apparatus for measuring the degree of polarization, and its calibration by means of the light transmitted at varying incidence by a single glass plate, are described.

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